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14. ABSTRACT
The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint. A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. Key findings are isolation of MSCs and stimulation towards osteogenesis in vitro on an octacalcium phosphate scaffold showing cells populated the scaffold and calcium deposits demonstrated with von Kossa stains; 2) A degradable form of photochemically crosslinked PEG norbomene gel was formulated and growth factors (TGF beta) tethered to the polymer showing peri-cellular cartilage matrix around the encapsulated swine chondrocytes; 3) Formation of new cartilage matrix was demonstrated in vivo in mice using photochemically crosslinked gels and swine articular chondrocytes 4) Chondrocytes encapsulated in photochemically crosslinked hydrogels can survive the crosslinking and implantation process as shown in swine 5) Bone marrow derived mesenchymal stem cells from swine were encapsulated in alginate, stimulated with chondrogenic growth factors, and showed in vivo matrix productions

15. SUBJECT TERMS- cartilage, articular, collagen gel, poly(ethylene)iglycol, photochemical crosslinking

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1. INTRODUCTION

Injuries to the cartilage surfaces of joint are particularly problematic because, unlike bone and other vascular tissues comprising the joint, cartilage is avascular and possesses limited capacity for repair and self-regeneration. Consequently, injury to cartilage in the articulating joints from trauma results in scar formation and possible arthritic changes that can lead to pain, stiffness, and loss of structure and function [1-3]. These joint injuries not only limit physical activity and mobility of those afflicted, but the inability to move freely can cause deep psychological scars and loss of independence when individuals have to depend on family and healthcare providers for constant assistance to perform daily life functions. The level of functional capability in the injured limb and ultimate quality of life depend on the successful outcome of joint surface regeneration performed as a secondary procedure weeks or even months after the initial injury. The return of function and the probability of return to active duty rely on successful restoration of the entire joint including the articular surface, and therefore, joint function. Lesions in the joint surface are commonly treated with microfracture [4], autologous cell implantation (ACI) [5], or osteoarticular autograft transfer system (OATS) [6]. To date, however, the outcomes of many restorative procedures are very unsatisfactory and an improved method for joint repair is a clear unmet need in military medicine. **The goal of our research is to introduce a novel means to regenerate the articular cartilage and restore normal function of the joint.** A strategy that can generate durable hyaline articular cartilage, which will be predominantly type II collagen, and is capable of integrating with the surrounding cartilage matrix (without fissures) could improve the long-term outcome of joint surface repair. *The scope of this research is to develop regenerative medicine approaches involving biocompatible hydrogel scaffolds seeded with autologous cells that provide three-dimensional environments favorable for promoting chondrogenesis for joint surface repair*.[7-10]

2. KEYWORDS

Cartilage; articular; collagen gel; poly(ethylene)glycol gel; photochemical crosslinking

3. OVERALL PROJECT SUMMARY

In the previous annual reports covering the first 36 months of this 48 month project, we reported on generating two candidate photochemically crosslinked hydrogels for encapsulating chondrocytes or chondrocyte precursor cells. The algorithm for making and testing the gels is presented in Figure 1. If the gels perform well in making neocartilage in mice, we would then proceed to swine studies. If not, our plan would be modified to re-examine cartilage production in vitro and in mice before proceeding. Photochemically crosslinked gels were made using collagen as a natural protein gel and poly(ethylene) glycol thiol-ene gels as a synthetic gel. The photochemically crosslinked collagen gels demonstrated increased resistance to collagenase digestion over uncrosslinked gels, but had little effect on changing the bulk modulus (stiffness) of the gels. This work is published in the Journal of Biomedical Materials Research Part A (Omobono et al., J Biomed Mater Res A. 2014 Jul 7. doi: 10.1002/jbm.a.35266. [Epub ahead of print]) (Appendix1). Testing of the PEG thiol-ene gels showed changes in the shear moduli were related to the weight percent of the gels. In vitro studies showed good viability of swine articular chondrocytes following the polymerization process. Modifications were made to the gel to tether TGF-β1 to the molecular backbone to promote matrix formation by encapsulated chondrocytes. This work is published in the Journal of Biomedical Materials Research Part A (Sridhar et al., J Biomed Mater Res A. 2014 Feb 26. doi: 10.1002/jbm.a.35115. [Epub ahead of print]) (Appendix 2). Results reported in year 2

suggested that the size of the original defects (6.5 mm) was too large to permit satisfactory regeneration of the joint surface. A pilot study using collagen gels defects measuring 1-2 mm in diameter in swine was reported for year 3 showing new cartilage formation in the defects and filling the lesions in the articular cartilage. Work in year 4 and presented in this report builds upon the in vivo work with photochemically crosslinked collagen and fibrin gels showing cartilage formation in mice. The collagen gel formulation has been pilot tested in two swine in year 4 with favorable outcomes showing new cartilage formation in the defects using cells in gels compared to empty defects and defects with gels alone. A new photochemically crosslinkable gel formulation, gelatin methacrylamide, was tested in vitro and in vivo in mice in year 4 showing cell survival and new cartilage matrix formation in vivo using both chondrocytes and mesenchymnmal stem cells. These results are presented in this annual report.

This annual report covering the fourth 12 months of the project reviews our progress testing the physical and chemical properties of these photochemically crosslinked hydrogels and their ability to promote chondrogenesis. The tasks referenced below pertinent to Year 4 under Task 1 include subtasks 1.1.b, 1.1.c, 1.2.a, and under Task 2 include subtask 2.1.a. A no-cost extension has been requested to continue the swine studies using favorable gel combinations.

Task 1 Test of photochemically crosslinked gels to produce cartilage and bone using chondrocytes and osteoblasts

Subtask 1.1.b Perform implantation of photochemically crosslinked collagen and PEG gels with chondrocytes and osteoblasts

- In vitro preparation and testing of chondrons capped with photochemically crosslinked gels.

Results: Although the previous design of photochemically crosslinked collagen, dual crosslinked collagen gels, and fibrin gels yielded some cartilage production in vivo, the results continued to yield inconsistent results in mice. The photochemical gels were performing as expected with sufficient polymerization and stability and we achieved good cell viability. However, the noecartilage that formed was fragmented and not sufficient for moving into large animals. Thus, the model was re-evaluated again to try to generate durable, contiguous cartilage matrix. Prior to initiating in vivo mice studies, we sought to refine the culture of the cells. The method was changed from encapsulating dispersed individual cells in the gel to placing an adherent collection of cells, referred to as a chondron, in the gel. A cell suspension of $10x10^6$ swine chondrocytes were placed in a 15mL conical tube and cultured in a horizontal motion in vitro for 14 days. During this time, the cells adhere together forming chondrons (Figure 2). The chondrons were then removed from the conical tube, placed in the center of a devitalized swine cartilage ring, and capped with a test hydrogel. After histological processing, H&E and Safranin-O stain showed that new cartilage matrix began to form during the 10 days in culture (Figure 3). Once this new method was established, we proceeded with in vivo studies in mice in subtask 1.1.c. described below.

- In vitro preparation and testing of a cell-mediated degradable PEG hydrogel that promotes articular cartilage extracellular matrix production

Previous work reported in year 3 demonstrated that chondrocyte ECM production is enhanced in the presence of locally-delivered tethered TGF-β1 in a non-degradable PEG network; however, matrix deposition was limited to the pericellular space (Appendix 3). In follow up work in year 4, our colleagues subcontracted at the University of Colorado used tethered TGF-β1 in a cell-mediated degradable system to promote cartilage ECM production and permit diffusion of these molecules throughout the scaffold. Degradation of collagen is the rate-limiting step in cartilage remodeling because it is the most abundant component of the ECM. Therefore, we used a collagenase-sensitive peptide linker derived from collagen, KCGPQG*IWGQCK (where the asterisk indicates the cleavage site), developed by Hubbell et al.[11] Previously, the Hubbell group encapsulated chondrocytes in a PEG gel linked with this peptide and found increased gene expression of cartilage matrix molecules compared to non-degradable gels. In order to catalyze the pericellular degradation process, we co-encapuslated mesenchymal stem cells (MSCs) with chondrocytes to aid in scaffold remodeling. In this work, we report the development of a MMP-sensitive PEG based hydrogel that employs co-culture of MSCs and chondrocytes to show that local degradation facilitates diffuse ECM deposition. TGF-β1 was thiolated and tethered into a PEG thiol-ene hydrogel since it has been shown to encourage cell matrix deposition of both chondrocytes and MSCs. We seeded MSCs at a low density to provide ample degradation of the linker while allowing us to focus on the ECM secretion of the chondrocytes. The results showed a cellularly-degradable scaffold that increases its mechanical integrity while permitting greater cartilage matrix production, that resembles the matrix of articular cartilage, throughout the gel relative to non-degradable scaffolds. In summary, we have developed a cell-mediated degradable hydrogel system that promotes diffuse cartilage ECM production which ultimately leads to constructs with improved mechanical properties over just 14 days. The system utilized the synergistic effects of co-culture between MSCs and chondrocytes to facilitate degradation of a collagen-derived, MMP-degradable peptide sequence as well as to promote cartilage ECM production. Additionally, we covalently tethered TGF-β1 to boost cartilage ECM production and confirmed that both encapsulated cell types maintain high viability and a spherical morphology in the gels. Furthermore, we found that the ECM generated resembles articular cartilage with both collagen typing by immunofluorescent staining (high type II collagen: type I collagen ratio) as well as Raman spectral analysis comparing the biochemical composition of constructs to that of native cartilage. Local degradation played a very important role in matrix elaboration with tissue engineering constructs since non-degradable constructs of the same formulation had significantly less ECM production and lower moduli values over 14 days. This PEG hydrogel system may have further applications as a scaffold for *in vivo* cartilage regeneration.

Subtask 1.1.c Evaluate cartilage and bone matrix produced in vivo in mice.

- Cells capped with photochemically crosslinked gels implanted in mice for matrix production in vivo.

Results: After encouraging results from the in vitro work presented above in subtask 1.1.b with chondrons, two pilot studies were completed with chondron constructs implanted into mice. The first study aimed to determine the ideal growth time of chondrons in vitro prior to implantation into mice. A cell suspension of $10x10^6$ swine chondrocytes were placed in a 15mL conical tube and cultured in a horizontal motion in vitro for 7 days, 14 days, and 21 days. After the desired growth time, each chondron was placed in the center of a devitalized ring (to simulate an actual knee cartilage defect) , gel was placed on top, and the construct was immediately implanted into nude mice. New cartilage

matrix was formed in all culture time points after 3 weeks in vivo (Figure 4). The 14 day culture time appeared hypercellular compared to 21 days and showed more consistent matrix formation than 7 days so it was selected as the ideal growth time for the chondrons in vitro. The second pilot study compared three different gel types – fibrin, collagen crosslinked (collagen+hv), collagen non crosslinked (collagen-hv) – to determine if the gel type increased the cartilage matrix production. The same method was followed and chondrons were cultured for 10 days prior to implantation into mice. After 3 weeks in vivo, each type of gel showed matrix production and there did not appear to be significant differences in cartilage formation (Figure 5).

A full study was completed using the same model (devitalized cartilage ring, immediate implantation). The chondrons were cultured for 14 days in vitro and the fibrin, photochemically crosslinked collagen, and non-crosslinked collagen gels were repeated for 6 and 12 weeks in vivo. Chondrons seeded without horizontal motion then capped with fibrin gel and chondrocytes encapsulated directly into fibrin gel were also done as controls. Figure 6 shows the gross images of the collagen crosslinked constructs after 6 and 12 weeks in vivo. New cartilage formation formed in vivo in mice after 6 and 12 weeks in all groups (Figures 7 and 8). Qualitative analysis of the histology images is inconclusive in determining which gel increased matrix formation. Biomechanical and biochemical results comparing the neocartilage from all groups are pending at the time of this report.

Subtask 1.2.a Initiate 3-month pilot study in swine with photochemically crosslinked collagen and PEG gels with bilayer of chondrocytes and osetoblasts

- Cells were encapsulated in photochemically crosslinked gels and implanted in swine to evaluate the survival of the cells in vivo.

Result: We previously reported in year 2 that a short-term pilot study confirmed that the cells survive the photochemical gelation and implantation process. However, the size (6.5 mm diameter) of the defects was deemed too large to get any meaningful regeneration of the articular cartilage surface. Since we are unable to limit weight bearing (as would be done in humans) in the swine, it is possible that the failure to regenerate full may be related to the significant mechanical forces on the defects in the joint. In consultation with our clinical collaborators, we decided to decrease the size of the defects to 1-2 mm in two swine done in year 3 of the grant. Instead of 6 large defects in the trochlear groove and the condyle of the femur, small 1-2 mm defects are made in the cartilage of the joint surface (Figure 9). These were either left empty, filled with gel alone, or treated with collagen gel containing chondrons. Empty defects and those with gel alone showed clefts in the cartilage surface at the time of harvest confirming that these treatments resulted in lesions that do not heal. Those defects treated with the collagen gel containing cells showed early cartilage formation in the defects, and the matrix stained positive for GAG production as demonstrated with Safranin O staining (Figure 10). A no-cost extension has been requested to complete the 3, 6, and 12 month animals in subtasks 1.2.a, 1.2.b, and 1.2.c, respectively.

Task 2 Stimulation of chondrogenesis by stem cells in photochemical gels

Subtask 2.1.a Perform initial study of collagen and PEG gels with stem cells implanted in mice

- Cells encapsulated in phtochemcially crosslinked GelMA (gelatin methacrylamide) implanted in mice for matrix production in vivo

Articular chondrocytes and mesenchymal stem cells were harvested from swine knee and bone marrow separately. The chondrocytes were grown in standard high glucose DMEM based chondrogenic media until 90% confluence, and the BMSCs were grown in articular chondrocyte conditioned media until P3 generation. The chondrocytes or BMSCs were suspended in the GelMA hydrogel at a concentration of 40 x 10^6 cells/ml, and then the cylindrical cell/GelMA samples of 5 mm height and 6mm diameter were UV-crosslinked for 15 minutes at an intensity of 180 mW/cm^2. All of the samples were precultured in the incubator for 2 weeks before implantation to mice. The cell viability was tested at 24 hours and 14 days after crosslinking (Figure 11). All of the in vivo samples were harvested at 6 weeks from mice for histological (Figure 12) and biochemical analyses (Figure 13). The neocartilage matrix was confirmed based on the achieved results. A repeated full study up to 12 weeks is currently underway.

4. KEY RESEARCH ACCOMPLISHMENTS

- In vitro preparation and testing of chondrons capped with photochemically crosslinked gels.
  - Showed that chondrons formed when the cells were placed in dynamic culture
  - Chondrons generated extracellular matrix during dynamic in vitro culture

- In vitro preparation and testing of a cell-mediated degradable PEG hydrogel that promotes articular cartilage extracellular matrix production
  - A degradable form of photochemically crosslinked PEG norbornene gel was formulated and growth factors tethered to the polymer
  - Pericellular cartilage matrix was demonstrated around the encapsulated swine chondrocytes
  - The addition of MSCs aided in the degradation of the hydrogel

- Cells capped with photochemically crosslinked gels implanted in vivo in mice showed new cartilage matrix
  - Chondrons were grown in vitro for 7, 14, or 21 days in vitro and were placed into rings of native cartilage and implanted into mice
  - Chondrons were combined with fibrin, collagen crosslinked (collagen+hv), collagen non crosslinked (collagen-hv) and implanted in vivo in mice
  - Both studies showed reliable formation of contiguous cartilage inside the native cartilage ring

- Cells were encapsulated in photochemically crosslinked gels and implanted in swine to evaluate the survival of the cells in vivo in swine knee cartilage defects
  - The size of the defects was 2 mm in diameter
  - Cartilage formed and filled the defect in specimens treated with chondrons and gel; not in the empty defects or those treated with gel alone

- Cells were encapsulated in phtochemcially crosslinked GelMA (gelatin methacrylamide) implanted in mice for matrix production in vivo
  - Cell viability of both chondrocytes and MSCs were >85% in the photopolymerized GelMA
  - The cells were capable of producing cartilage extracellular matrix when the gels were implanted into mice
  - The cells produced GAGs and collagen type II typical of hyaline cartilage in this gel

5. CONCLUSIONS

At the conclusion of the fourth year of this grant award, we made modifications to the formulations of photochemically crosslinked hydrogels that will be used to deliver chondrocytes to articular cartilage defects in the swine knee joint. As we described in our testing paradigm shown in Figure 1 of this report, gels that do not perform well in vitro or in vivo in mice will be reformulated and tested before moving into large animal swine studies. With the modifications described in this report, we initiated large animal testing in year 4.

Although the photochemically crosslinked hydrogels performed well in vitro and made cartilage in vivo in mice, some failed to produce adequate extracellular matrix to be used in our swine knee defect model. The gels demonstrate good cell viability, but the matrix that formed often had gaps between the individual cells. We explored bioreactors to address this and developed a dynamic culture method that produced collections of cells—chondrons—that performed better in making contiguous cartialge matirx in a reliable manner. This approach was tested in vitro and in vivo in mice. These results demonstrated that this might be a good solution to problems we had generating good cartilage matrix in swine. Another problem was the proposed size of the cartilage defects in the swine. The original research plan involved making several 6.5 mm osteochondral defects in the trochlear groove and the condyles of the distal femur. We found, however, that the sizes of these defects were too large to permit regeneration of the cartilage surface using any of the gels that we have developed. A pilot study in year 3 reduced the size of the lesions to 1-2 mm with better evidence of regeneration. Results from a pilot study in 2 swine showed that cells encapsulated in collagen gels regenerated the articular cartilage in the swine. A no-cost extension has been requested to complete the 3, 6, and 12 month animals in subtasks 1.2.a, 1.2.b, and 1.2.c, respectively.

Building on our results from year 3 in which we developed a photopolymerizable PEG thiol-ene hydrogel as a platform to covalently attach a growth factor to provide a local and sustained presentation to chondrocytes for cartilage tissue engineering applications, work in year 4 examined a means to degrade the polymer using a combination with stem cells. The results showed a cellularly-degradable scaffold that increases its mechanical integrity while permitting greater cartilage matrix production that resembles the matrix of articular cartilage, throughout the gel relative to non-degradable scaffolds.

The goal of task 2 was to isolate bone marrow derived mesenchymal stem cells from swine and encapsulate these cells in the photochemical gels. Studies in year 3 reported on encapsulating BM-MSCs in the hydrogels and testing these differentiated cells in gels in vivo in mice. New studies in year 4 have demonstrated that photochemiclly crosslinkable gelatin methacrylamide can support cell encapsulation and matrix formation. These results with MSCs are encouraging and will be pursued during the period of no-cost extension.

"So What" Section:

In the first through third years of this project our team developed photocrosslinkable hydrogels that could be used to regenerate cartilage in defects in the articular cartilage surface of joints with the goal of restoring normal joint function. These crosslinkable hydrogels serve as biomimetic polymers that provide a favorable environment for encapsulating chondrocytes (the native cell type found in cartilage) and chondrocyte precursor cells (mesenchymal stem cells). We have successfully formulated collagen and PEG gels, and now a new gelatin hydrogel. Whereas we had intended to complete a 3-month study in swine in year 3, the mechanical and biological characteristics were inferior and not suitable for implantation in large animals. According to our iterative testing protocol, work in years 2 and 3 focused on improving the mechanical and biological characteristics of these gels. A pilot

study in swine has demonstrated proof of principle that this technology permits cell survival during the photochemical crosslinking process and implantation in swine in the short term (i.e., up to 2 weeks). Nonetheless, the size of the cartilage defect proposed in the original application appears to be too large to permit successful regeneration of the joint surface. To resolve this obstacle, we reduced the size of the defect to 1-2 mm in diameter and performed a pilot study in two swine. These results showed regeneration of the articular cartilage. We anticipate that this new modification along with changes made in gel formulation and polymerization will be useful adjuncts for joint surface repair and regeneration. A no-cost extension has been requested to complete the 3, 6, and 12 month animals in subtasks 1.2.a, 1.2.b, and 1.2.c, respectively.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Abstracts presented:
Cellularly Degradable PEG Hydrogels with Tethered TGF-β1 for Improved Cartilage Engineering. Sridhar BV, Randolph MA, Anseth, KS. 2014 Society for Biomaterials Annual Meeting April 16-19, Denver, CO

Photochemical Crosslinking Stabilizes Protein Hydrogels for Articular Cartilage Regeneration. M. Randolph, M.A. Omobono, X. Zhao, S. Jang, R.W. Redmond, T.J. Gill; Jury decision: Accepted
Session: 9.40 - Biomaterials & Scaffolds (Abstract No. 5771), International cartiagle repair Society, Izmir, Turkey September 2013

Published manuscripts:

Sridhar BV, Doyle NR, Randolph MA, Anseth KS. Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production. J Biomed Mater Res A. 2014 Dec;102(12):4464-72. doi: 10.1002/jbm.a.35115. Epub 2014 Feb 26.

Omobono MA, Zhao X, Furlong MA, Kwon CH, Gill TJ, Randolph MA, Redmond RW. Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration. J Biomed Mater Res A. 2014 Jul 7. doi: 10.1002/jbm.a.35266. [Epub ahead of print]

Manuscript drafted for submission to *Biomaterials*:
Sridhar BV, Jain R, Brock JL, Silver JS, Randolph MA, Leight JL, Anseth KS. Development of a cell-mediated degradable PEG hydrogel that promotes articular cartilage extracellular matrix production.

7. INVENTIONS, PATENTS AND LICENSES:

None to report

8. REPORTABLE OUTCOMES FOR YEAR 4

Manuscript drafted for submission to *Biomaterials*:
Sridhar BV, Jain R, Brock JL, Silver JS, Randolph MA, Leight JL, Anseth KS. Development of a cell-mediated degradable PEG hydrogel that promotes articular cartilage extracellular matrix production.

9. OTHER ACHIEVEMENTS:

None to report

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11. SUPPORTING DATA

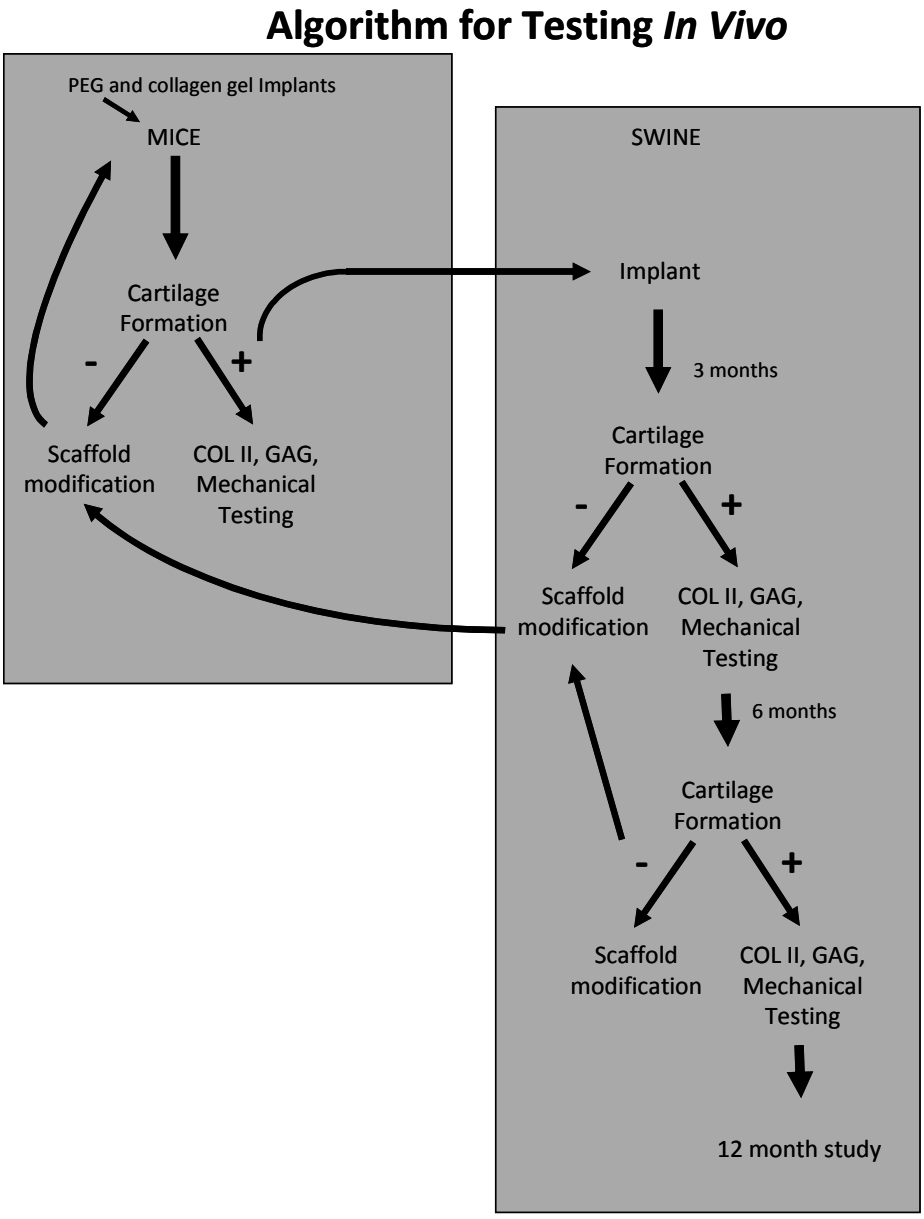
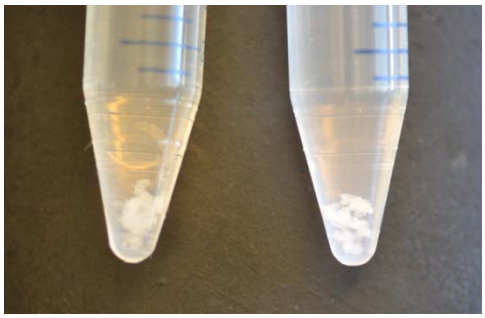
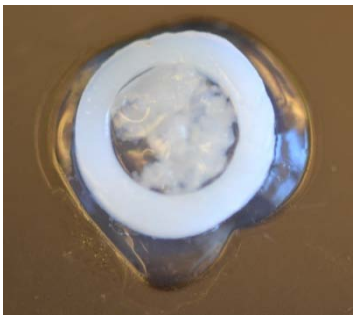


Figure 1. This is the testing paradigm proposed in the original grant application. The gels are made and tested in vitro and in vivo in mice (left box) prior to embarking on the large animal swine model (right box). If gels fail to perform in the mice they are not tested in swine. Additionally, if the gels should perform poorly in the early test phase in swine, the gels are reformulated and tested again in mice before re-embarking on the swine studies. Many changes outlined in this report describe changes to the gels so that large animals are not used unnecessarily for gels that have not been optimized.

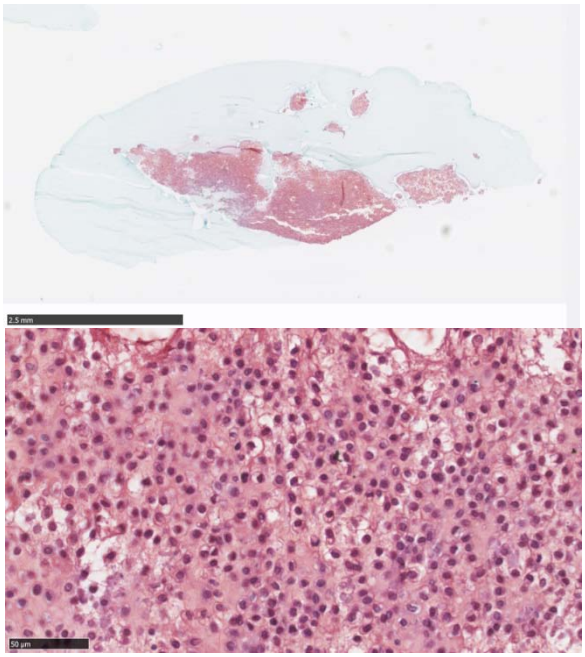


chondrons in 15mL conical tube

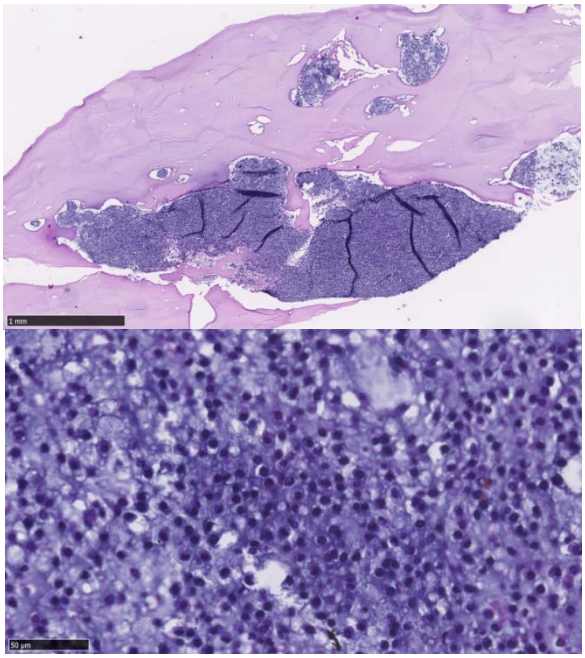


chondron capped w/ fibrin gel in ring

Figure 2. Swine chondrocytes formed into an adherent collection of cells referred to as chondrons after 10 days of culture in vitro (left). The chondrons were placed into the center of a center of a native cartilage ring at which time the hydrogel was added and photopolymerized (right).



Safranin-O



H&E

Figure 3. Chondrons that were grown in dynamic culture for 10 days andimmediately placed in fibrin gel only for histological processing. The red-stained areas in top left frame stained with Safranin-O and the blue-stained areas in the top right stained with hematoxyline and eosin show the aggregates of cells that form during the culture period. The frames on the bottom left and right are higher magnification images showing the cell in the chondrons.

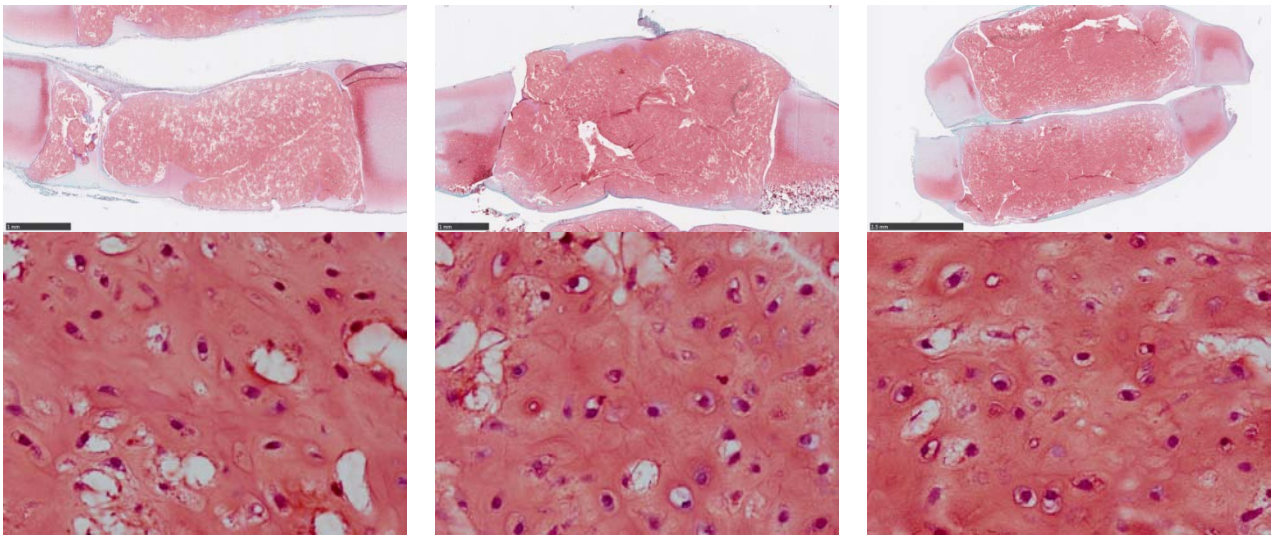


Figure 4. Safranin-O staining of the chondrons placed in dymanic culture for 7, 14, and 21 days, capped with fibrin gel, and placed in vivo in mice for 3 weeks. (Top row 1mm scale bar, Bottom row 400x)

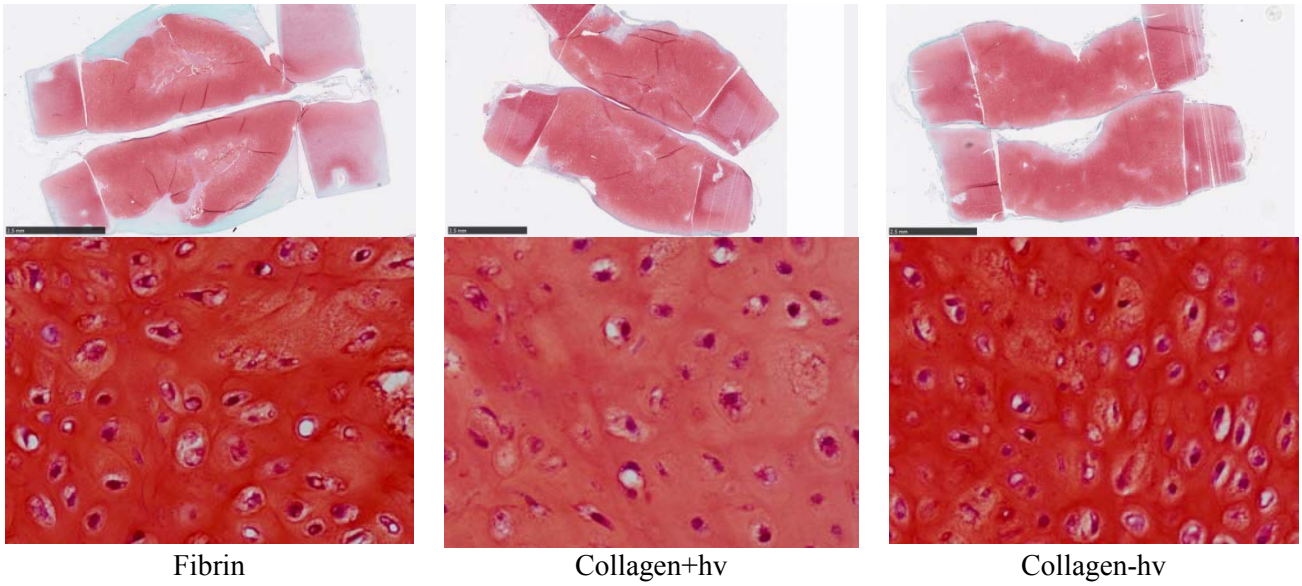


Figure 5. Safranin-O staining of the chondrons placed in dynamic culture for 10 days in vitro, capped in fibirn gel , collagen gel, or photochemicaly crosslinked collagen gel, and placed in vivo in mice for 3 weeks. (Top row 1mm scale bar, Bottom row 400x)

Figure 6. Appearance of chondrons capped with collagen+hv gel when harvested at 6 and 12 weeks.

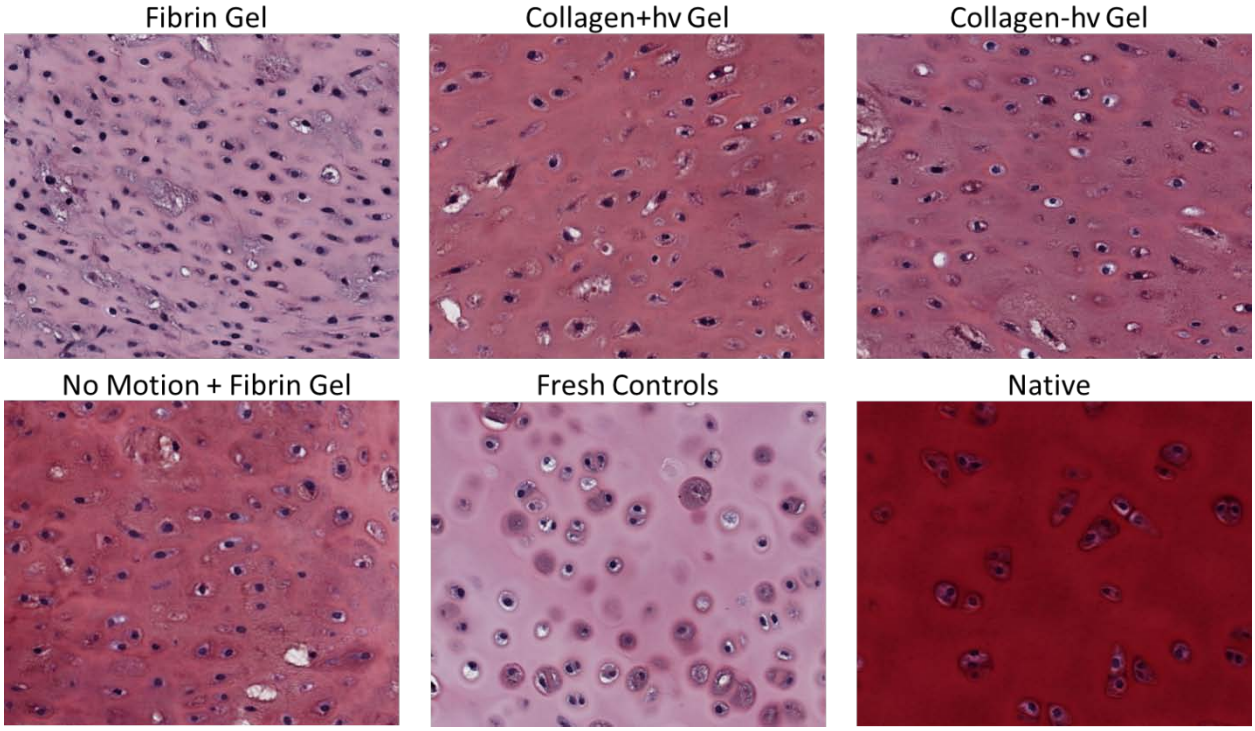


Figure 7. Safranin-O staining of all 5 experimental groups after 6 weeks in vivo and native cartilage. (All 400x)

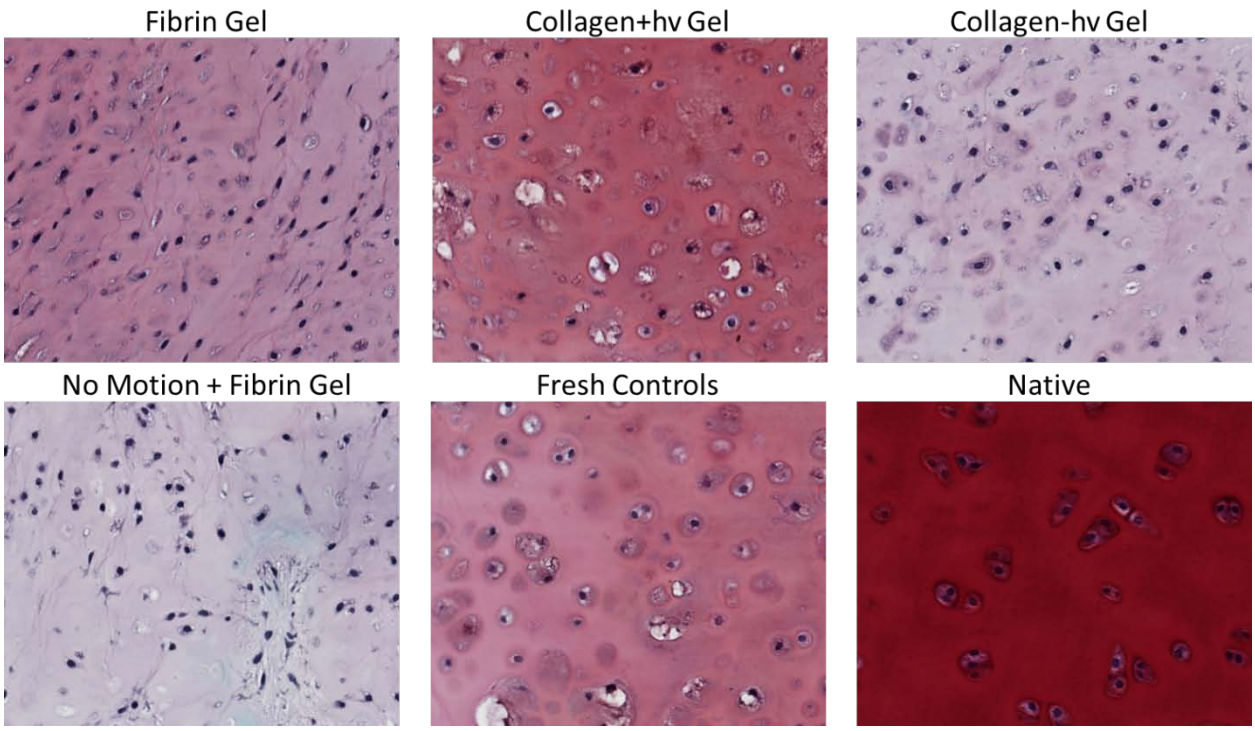


Figure 8. Safranin-O staining of all 5 experimental groups after 12 weeks in vivo and native cartilage. (All 400x)

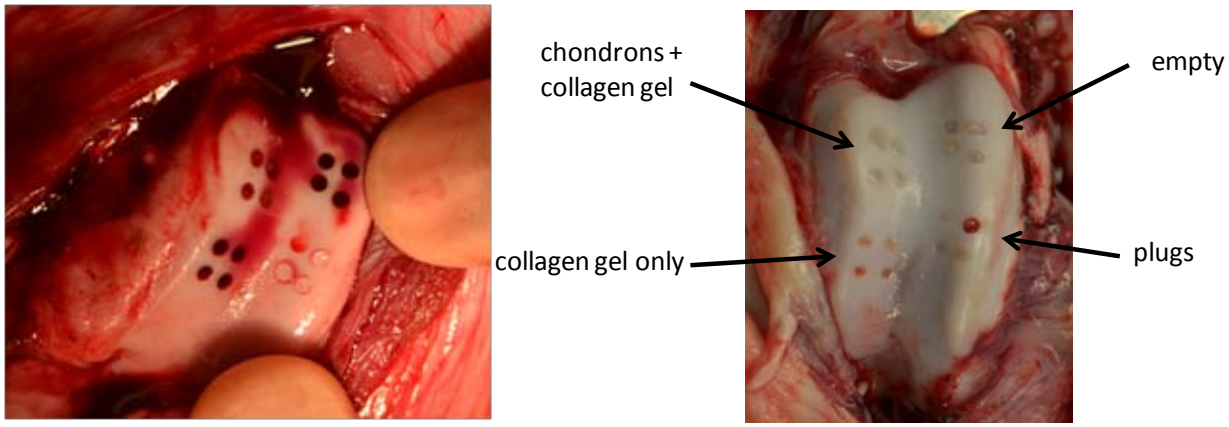


Figure 9. Surgery of the swine knee showing 2mm defects in groups of 4 in left image. Results after 6 weeks showing each of the treatment groups

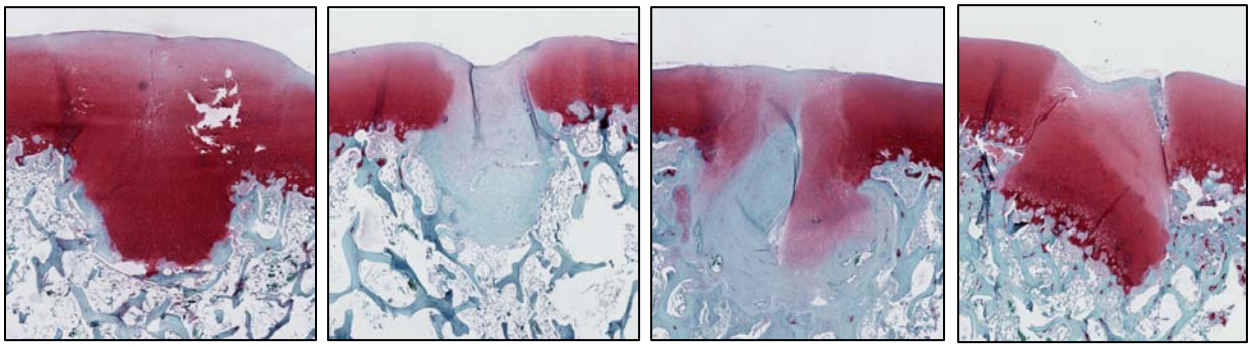


Figure 10. Cartilage generated in situ in swine knee using collagen gel and swine articular chondrocytes on left compared to defect treated only with collagen gel without cells (second from left), left empty (second from right) and control plugs placed back into the defect (right) (Safranin-O specimens stained for GAG).

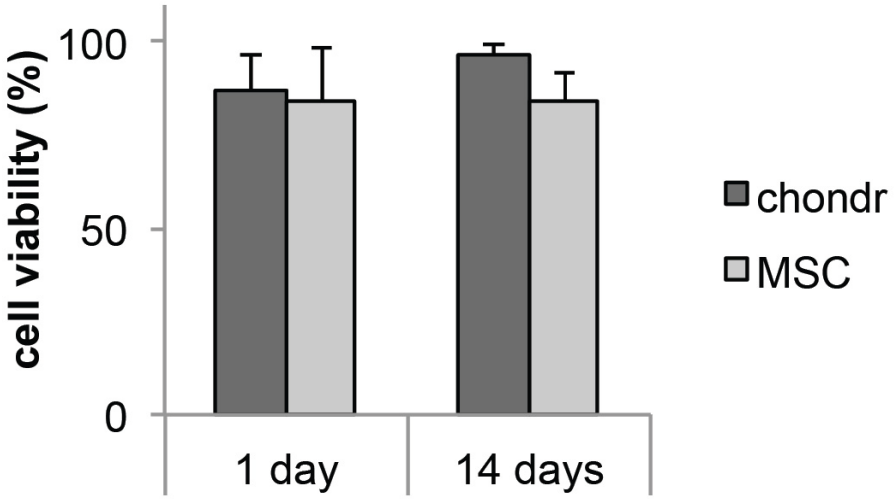


Figure 11. Cell viability at 1 and 14 days of in vitro culture in photochemically crosslinked gelatin methacrylamide gels showing viability was greater than 85%.

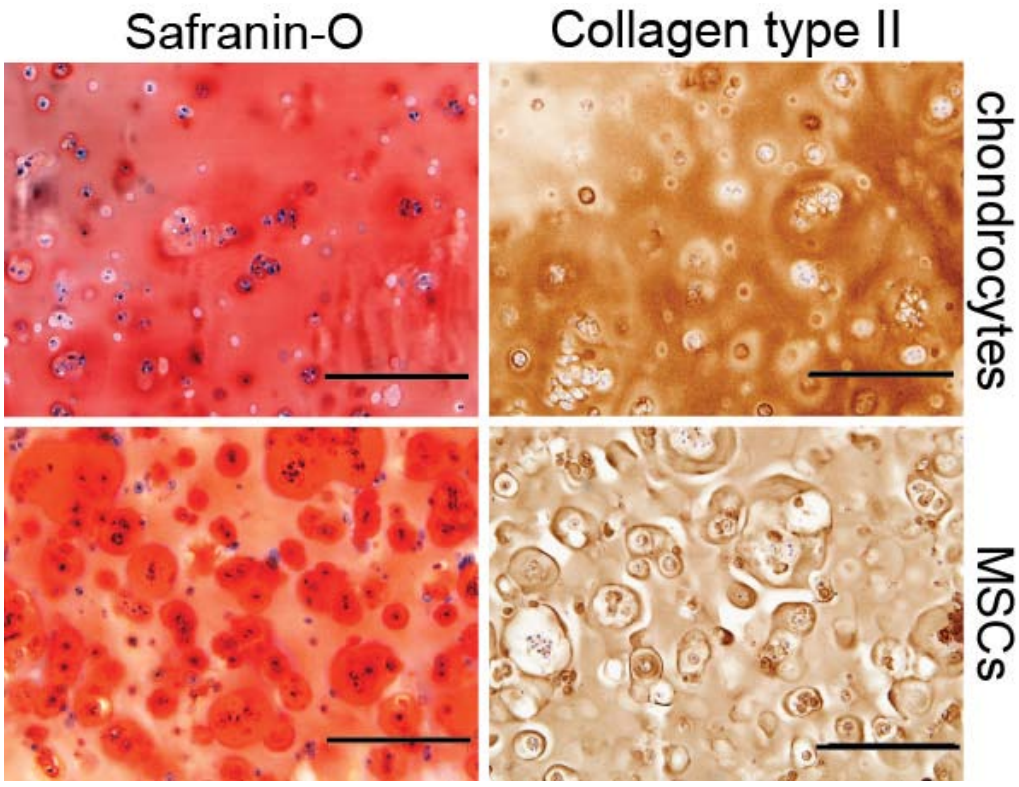


Figure 12. Results from swine chondrocytes and swine mecenchymal stem cells encapsulated in photochemically crosslinked gelatin methacrylamide and placed in vivo in mice for 6 weeks. The Safranin-O stained sections indicate GAG produced by both cells types in the pericellular areas. Type II collagen, typical of hyaline cartilage, was produced by both cell types.

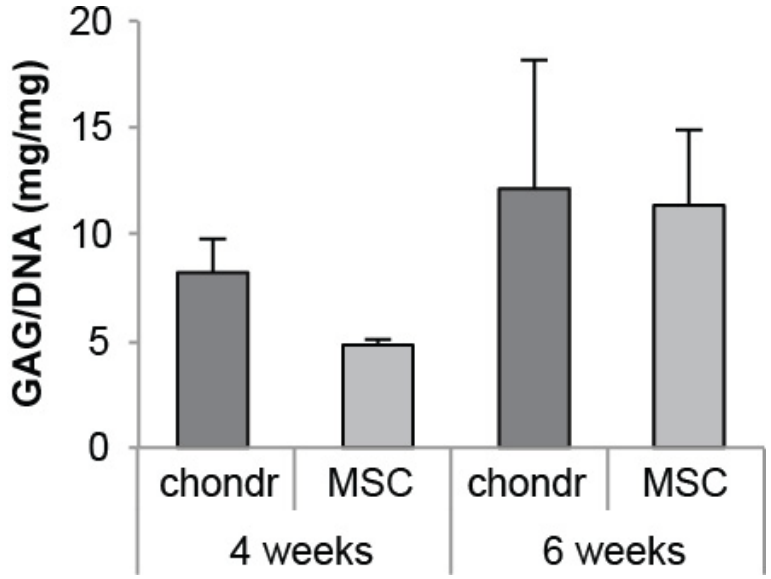


Figure 13. The amount of GAG per cell was higher by chondrocytes than the mesenchymal stem cells when placed inteh gelatin methacrylamide gels, but both cell types made typical GAG.

12. **APPENDICES**

Appendix 1: Omobono MA, Zhao X, Furlong MA, Kwon CH, Gill TJ, Randolph MA, Redmond RW. Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration. J Biomed Mater Res A. 2014 Jul 7. doi: 10.1002/jbm.a.35266. [Epub ahead of print]

Appendix 2: Sridhar BV, Doyle NR, Randolph MA, Anseth KS. Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production. J Biomed Mater Res A. 2014 Dec;102(12):4464-72. doi: 10.1002/jbm.a.35115. Epub 2014 Feb 26.

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Enhancing the stiffness of collagen hydrogels for delivery of encapsulated chondrocytes to articular lesions for cartilage regeneration

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Abstract: This study investigated a dual crosslinking paradigm, consisting of (a) photocrosslinking with Rose Bengal (RB) and green light followed by (b) chemical crosslinking with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysuccinimide (NHS) to enhance collagen gel stiffness. In group 1, 50 μL collagen constructs of 2% (w/v) type I collagen containing 10 μ*M* RB were allowed to gel spontaneously at 37°C. In group 2, the spontaneous gels were exposed to green light (532 nm). In group 3, the photochemically crosslinked gels were subsequently treated with a 1-h exposure to 33 m*M* EDC/6 m*M* NHS. Samples ($n = 18$) were subjected to 0.08% (w/v) collagenase digestion, and the storage modulus of samples was measured by rheometry. Viability of encapsulated chondrocytes was measured by live/dead assay. Chondrocytes were ≥95% viable in all constructs at 10 days in vitro. Resistance to collagenase digestion increased as; spontaneous gels (2 h) < photochemical gels (3–4 h) < dual crosslinked gels (>24 h). The storage modulus of dual-crosslinked constructs was increased 5-fold over both photocrosslinked and spontaneous gels. As the dual crosslinking paradigm did not reduce encapsulated chondrocyte viability, these crosslinked collagen hydrogels could be a useful tool for the practical delivery of encapsulated chondrocytes to articular defects. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: collagen, cartilage tissue engineering, crosslinking, hydrogel, scaffold

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INTRODUCTION

Articular cartilage has little innate ability to heal and presents significant clinical challenge for natural regeneration. It is populated exclusively by chondrocytes,[1] but is avascular and lacks the capacity for complete, spontaneous regeneration in response to any focal injury to the contiguous extra-cellular matrix (ECM). Any procedure aimed at restoring the articular surface is required to (a) create natural hyaline cartilage to fill the defect and (b) allow integration of the neocartilage with existing healthy cartilage.

Among many potential natural and synthetic matrices in tissue engineering, collagen has proven to be useful in hydrogel,[2,3] membrane,[4,5] and porous scaffold[6,7] forms. Collagen is naturally occurring in human tissue, including articular cartilage.[8] Collagen molecules self-assemble into a hydrogel matrix at physiological pH and temperature via hydrogen bonding but these spontaneous hydrogels are weak in mechanical integrity and are rapidly digested by enzymatic attack. In an attempt to overcome these drawbacks, we exposed collagen gels containing photoreactive dyes to visible light to induce crosslinking of adjacent collagen molecules. These photocrosslinked gels were protected from contraction due to cellular interaction with the matrix[9] and chondrocytes encapsulated in photocrosslinked collagen hydrogels formed hyaline-like cartilage.[10] However, these gels are relatively soft and difficult to handle, and the aim of this work was to modify the crosslinking process to provide a stiffer collagen hydrogel that would be more user-friendly in ultimate clinical use.

Any increase in gel stiffness must be achieved without affecting the viability of encapsulated chondrocytes or their

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TABLE I. Group Designations for EDC/NHS Concentration and Time of Exposure Study

Dilution [EDC]/ [NHS] (m*M*)	15-Min Exposure	30-Min Exposure	45-Min Exposure	60-Min Exposure
33/6	1–15	1–30	1–45	1–60
16.5/3	2–15	2–30	2–45	2–60
6.6/1.2	5–15	5–30	5–45	5–60
3.3/0.6	10–15	10–30	10–45	10–60

This work was presented at the Annual Meeting of the Orthopedic Research Society, San Antonio, TX, in February 2013.

ability to remodel the existing type I collagen scaffold and generate neocartilage. The feasibility of implanting a collagen hydrogel in an articular surface defect hinges on the mechanical integrity of the hydrogel during implantation and early repair. During this early period chondrocytes must have a scaffold that supports viability and permits new cartilage formation. Photocrosslinked gels may lack the stiffness necessary to withstand extended mechanical loading and an enhancement in construct stiffness could be beneficial.

Chemical crosslinking using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) is nontoxic and useful for crosslinking collagen in tissues.[11–15] EDC, in the presence of NHS, activates carboxyl groups of aspartic and glutamic acid residues of collagen to react with nucleophilles, such as primary amines (lysine and hydroxylysine) and hydroxyl groups, to create zero-length crosslinks.[16] Treating collagen hydrogels with EDC/NHS is a potential method for increasing crosslinking, and the combination of photo- and chemical crosslinking could yield a hydrogel with improved stiffness.[17]

In this study, we have used dual-crosslinking (photochemical + chemical) of type I collagen hydrogels to test whether the stiffness of collagen gels can be substantially increased to make them useful for articular cartilage defect repair. Effects on mechanical properties of the gel, encapsulated chondrocyte viability in vitro, and resistance to collagenase digestion were evaluated to determine the potential of this new crosslinking paradigm for articular cartilage regeneration.

MATERIALS AND METHODS

Materials

Rat tail type I collagen (11.4 mg/mL) was obtained from BD Biosciences, Bedford, MA. Ham F12, 10% fetal bovine serum. 1% antibiotic/antimycotic liquid and 1% MEM nonessential amino acids were all from Gibco (Carlsbad, CA). Sodium hydroxide (NaOH), Rose Bengal (RB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide and N-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma-Aldrich (Natick, MA). Type II collagenase at 245 U/mg was purchased from Worthington Biochemical (Lakewood, NJ).

Preparation of collagen hydrogels

Rat tail type I collagen was mixed in an Eppendorf tube with chondrocyte media [Ham F12, 10% fetal bovine serum (Gibco), 1% antibiotic/antimycotic liquid, 1% MEM nonessential amino acids], 50 m*M* stock NaOH, and 90 μ*M* Rose Bengal diluted in chondrocyte media. The collagen solutions were adjusted to pH 7.2 ± 0.2 using 50 m*M* NaOH. The final concentrations were 10 μ*M* Rose Bengal and 2.54 mg collagen/mL.

Typically, 50 μL of the hydrogel mixture was aliquoted into cylindrical molds of 4.7 mm diameter and spontaneous gelation of the collagen solution was induced by incubation at 37°C for 1 h. Photochemically crosslinked gels were prepared by exposure to green light from a continuous wave KTP laser (LRS-0532-PFH-000500-05, Laserglow Technologies, Canada, 800 mW, 532 nm) at 20 J/cm^2 fluence from three different angles to assure equal illumination throughout the specimen. A group of photochemically crosslinked gels also underwent subsequent chemical crosslinking following ejection from the mold by immersion in a solution of 33 m*M* EDC, 6 m*M* NHS, in 50 m*M* HEPES buffer for 1 h. Another group of gels was prepared solely by exposure to EDC/NHS for 1 h without prior photochemical crosslinking.

To compare concentration and time of exposure to EDC/NHS solution, all collagen gels were prepared in a similar fashion through green laser exposure. Samples were then subjected to increasing dilutions and increasing times of exposure ($n = 3$ per group) according to Table I.

Collagenase digestion assay

Collagen hydrogel constructs were submerged in 10 mL of 0.08% w/v type II collagenase (in PBS) to determine the degree of protection from enzymatic degradation provided by crosslinking treatments in 15-mL conical tubes (BD Biosciences). Tubes were placed on a lab rocker at room temperature and checked every 15 min to determine the length of time for complete dissolution of the construct. Gels that were still intact after 24 h were recorded as "undigested". Three individual trials were conducted with $n = 6$ per trial for a total of $n = 18$ samples per group.

Mechanical testing

The viscoelastic storage modulus, G', of each collagen construct was measured using a rheometer (TA Instruments AR-G2, 8 mm Rough Steel Smart-Swap plate, part #511080.906). Samples were subjected to a frequency sweep from 1 to 10 radians/s with constant 2% strain rate and an 800-μm gap under continuous, room-temperature oscillation. Shear elastic moduli were calculated from the stress measured, using the equation;

$$G' = (\sigma_0/\varepsilon_0)\ \cos(\delta)$$

where σ_0 is the stress applied, ε_0 is the strain measured, and δ is the measured lag between phases. Three trials were conducted at $n = 6$ per group per trial for a total of $n = 18$ samples per group.

Chondrocyte viability

Knee joints were obtained from euthanized, 4-month-old Yorkshire swine and dissected under sterile conditions to expose the femoral condyles and the posterior face of the patella. The cartilage was dissected from the knee and

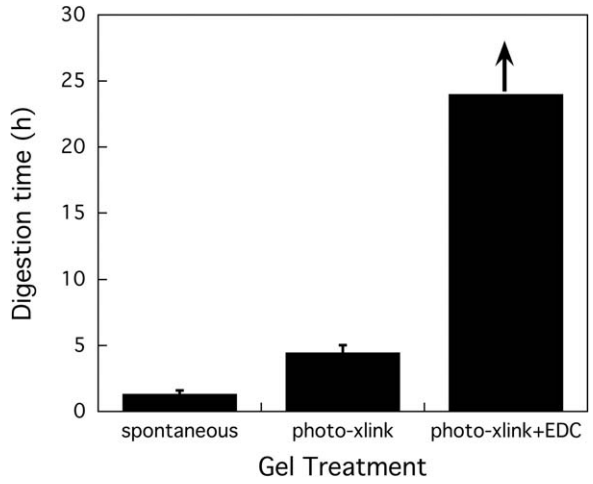

FIGURE 1. Times of degradation of 2% w/v collagen gels of various crosslinking treatments when exposed to 0.08% w/v collagenase enzyme at room temperature (n=6 per group). Photocrosslinked gels lasted significantly longer (3.46 ± 0.13 h) than spontaneous gels (1.32 ± 0.06 h) ($p < 0.0001$). Gels exposed to dual crosslinking were completely undigested after 24 hours.

digested in 0.1% w/v type II collagenase solution in Ham F-12 medium (Life Sciences, Grand Island, NY) containing 1% Anti-Anti (antibiotic, antimycotic, Life Sciences) overnight at 37°C. Digested cartilage solutions were passed through a 100-μm cell strainer and centrifuged at 250*g* for 10 min. The cell pellet was collected and washed twice with fresh chondrocyte media. Cells were counted on a hemacytometer and tested for initial viability using the trypan blue exclusion assay. Chondrocytes were plated in monolayer at a density of 2×10^6 cells/150 cm^2 and cultured at 37°C and 5% CO_2 until 80% confluent. Cultured plates were then exposed to 0.05% trypsin-EDTA (Invitrogen) and cells were collected in chondrocyte media, and washed twice with fresh media.

Cells were then suspended in collagen hydrogel mixture at 1.0×10^7 cells/mL hydrogel ($n = 4$). Gels were molded, incubated, and crosslinked using the dual-crosslinking approach. Photo-crosslinking was carried out as described above and the gels were then incubated in EDC/NHS (33/6 m*M*) in HEPES buffer for 1 h. After crosslinking treatment, gels were submerged in chondrocyte media for in vitro culture. After 10 days the gels were immersed in a solution of Live/Dead Viability Assay (Invitrogen, Grand Island, NY), containing 1.6 μ*M* calcein AM and 200 n*M* ethidium homodimer-1, for 1 h. The stained constructs were embedded in O.C.T. Compound (Tissue Tek), frozen at −20°C for 1 h, and sliced into 10-μm sections using a cryostat (Leica CM3050). These sections were then imaged using a Nikon Eclipse (TE2000U) fluorescence microscope using FITC (480ex/535em) and TRITC (535ex/610em) filters, and processed with NIH ImageJ software.

Statistics

Results of collagenase and mechanical testing data are reported as mean ± standard deviation. Significance was calculated using 1-way ANOVA analysis with Bonferroni's Multiple Comparison Test post-test with $p < 0.05$ considered significant. Percentage chondrocyte viability was calculated by counting dead (red) and live (green) cells from the fluorescent photographs. Samples were photographed in triplicate, capturing one central image and two boundary images per sample. Both live and dead cells were counted manually from which the viable fraction of cells was calculated. Manual counting was performed by five independent evaluators and results are presented as the mean of the five independent viability percentage for each gel ± standard deviation.

RESULTS

Resistance to collagenase digestion

Full degradation was defined as complete dissolution of the hydrogel by collagenase. The time to degradation (tdeg) of collagen hydrogels photocrosslinked with Rose Bengal and green laser exposure (3.5 ± 0.5 h) exhibited > 2-fold increase ($p < 0.0001$) in time of degradation from the spontaneous gels (1.3 ± 0.3 h). Dual-crosslinked constructs digested in collagenase solution exhibited no signs of degradation, even after 24 h (Fig. 1). Gels that were crosslinked with EDC/NHS alone for 60 min were soft but very resistant to degradation; after 24 h there were no signs of degradation. Exposure of constructs to different concentrations of EDC/NHS for different times showed varying degrees of resistance to digestion (Fig. 2). Within a treatment dilution group, t_{deg} increased with increasing time of exposure to EDC/NHS. Between treatment dilution groups, t_{deg} increased with increasing concentration of EDC/NHS.

Mechanical testing

Values for storage modulus in spontaneous control gels (25.8 ± 1.5 Pa) and storage modulus in photocrosslinked

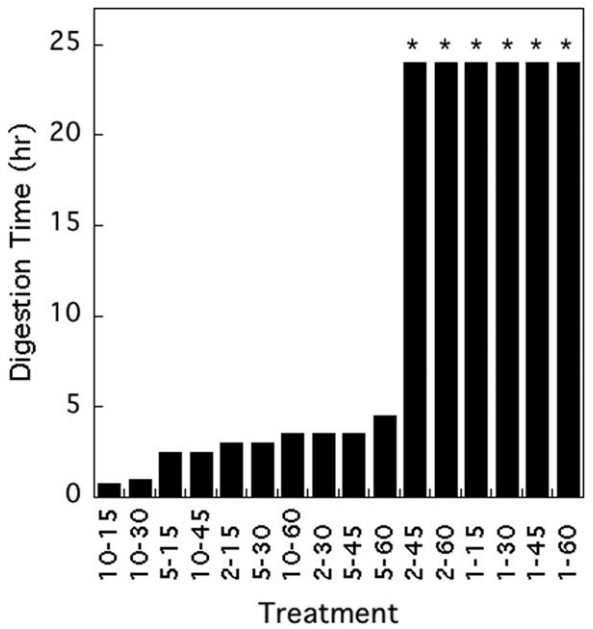

FIGURE 2. 0.08% w/v collagenase digestion of 2% w/v collagen gels treated with different dilutions and exposure times of EDC/NHS. Refer to Table I for group designations. Gels labeled with an asterisk (*) did not digest after 24 hours, when the study was capped.

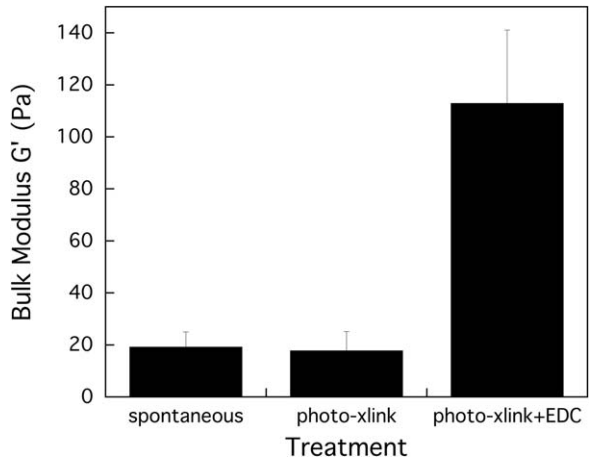

FIGURE 3. Storage modulus G′ as measured by a rheometer of 2% w/v collagen gels of various crosslinking treatments (n=6 per group). There was no significant difference between spontaneous gels (25.8 ± 1.5 Pa) and photocrosslinked gels (21.4 ± 1.8 Pa) ($p = 0.58$). There was a significant difference, up to a 5-fold increase in storage modulus when collagen gels were treated with dual crosslinking (117.6 ± 6.9 Pa) ($p < 0.0001$).

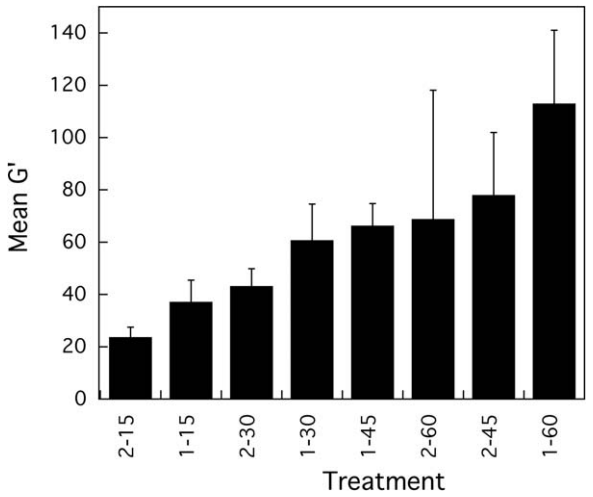

FIGURE 4. Storage modulus (G′) by rheometry of 2% w/v collagen gels treated with different dilutions and exposure times of EDC/NHS. Refer to Table I for group designations. There is no statistical significance between gels of groups exposed to lower concentrations of EDC/NHS. The first significant difference is noticed at group 2–30, with varying degrees of stiffness modulation between group 2–30 and maximum crosslinking treatment, group 1–60.

gels (21.4 ± 1.8 Pa) showed no statistically significant difference ($p = 0.07$). Storage modulus measurements for dual-crosslinked gels were 5-fold higher (117.6 ± 6.9 Pa) than both photocrosslinked and spontaneous gels ($p < 0.0001$, Fig. 3). Gels exposed to the most dilute concentrations of EDC/NHS (1 : 10, 1 : 5) did not show any significant difference in storage modulus from uncrosslinked and photocrosslinked groups but groups exposed to EDC/NHS diluted 1 : 2 for at least 15 min showed a trend of increasing storage modulus with increasing exposure time (Fig. 4).

Chondrocyte viability

Chondrocytes in six different gels treated with the dual-crosslinking approach exhibited $96.1 \pm 2.3\%$ viability in hydrogel implants after 10 days of in vitro culture (see Fig. 5 for an example). Cells residing in the peripheral regions of the constructs tended to have a lower viability than those in the central regions of the gels. Overall, the viability far exceeded our target viability of 90% for encapsulated chondrocytes to produce healthy ECM.

DISCUSSION

Articular cartilage regeneration is a challenging clinical problem. The newest tissue engineering-based therapy, autologous chondrocyte implantation (ACI), involves biopsy of healthy hyaline cartilage from a nonweight bearing area of the knee to harvest autologous chondrocytes for expansion in vitro followed by injection of cultured cells to the cartilage defect. The cells are typically suspended in saline and a periosteal flap is sutured to the cartilage surface. Not only does this repair require multiple surgeries to complete, but there is little data supporting the benefits of ACI versus microfracture[18] or OATS.[19] A common problem during this procedure is the leaking of cell-saline suspension from under the periosteal flap out of the target site due to compressive pressure despite sealing the surgical site with suture and fibrin glue.

Finding a suitable matrix to support cellular activity and ECM generation is a common problem in tissue engineering.[12,20–23] Native articular cartilage consists of different types of collagen fibrils, but is mostly type II collagen by dry weight.[8] Type I collagen is the main structural component in many native tissues in the body, therefore it is an attractive option as a nontoxic, biomimetic matrix to support natural ECM regeneration by chondrocytes. Collagen hydrogels can be formed at 37°C but these "spontaneous"

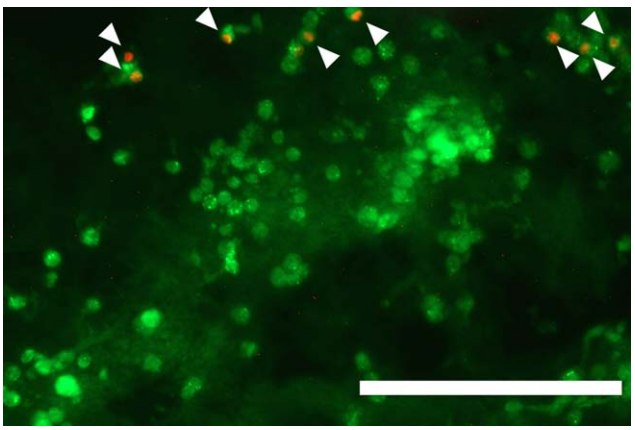

FIGURE 5. Live/Dead photographs of dual crosslinked construct after 10 days of in vitro culture. Pictures are taken at a height of $750\mu m \pm 50\mu m$ from the base of each construct in a $10\mu m$ cryotome slice. Dead cells are denoted by white arrows and scale bar is 300 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

gels are very soft and are unsuitable for implantation in focal articular cartilage defects.

Photocrosslinking of synthetic polymers typically is well-established and typically uses ultraviolet (UV) illumination of photoinitator compounds that react with synthetic monomers to cause chain reaction polymerization via radical reactions. However, for the purpose of cell encapsulation the UV illumination and the materials used would not be appropriate due to inherent cytotoxicity.[24] For this reason we have investigated visible light as the energy source for crosslinking along with nontoxic initiators. Under these milder conditions the initiating event can be electron transfer between excited initiator and monomer and/or energy transfer from photoinitator to dissolved oxygen to form singlet oxygen and its subsequent reactions (e.g., oxidation of histidine and reactions of photooxidized histidine with other amino-containing residues[25] to form a crosslink).[26]

We have previously shown that photochemical crosslinking of a collagen hydrogel provides a scaffold that supports encapsulated chondrocytes and stimulates cartilage-like ECM production.[9,10] Photochemical crosslinking certainly stabilizes the matrix and makes it more resistant to enzymatic degradation but provides little in the way of additional mechanical stiffness that would enhance practical handling of the gel and remove the need for a covering material to be affixed over the cartilage defect to prevent loss of unstable gel. Thus, we sought an improved crosslinking mechanism that enhances initial stiffness of these collagen-based hydrogels.

An obvious possibility would be to increase the fluence (photons/cm^2) incident on the gel. However, we have already shown in cell-free collagen gels that there is a plateau in the fluence dependence of crosslinking, suggesting a saturation of all sites that can be photochemically crosslinked, and further exposure does not equate to increased crosslinking. As an aside, this approach would also be complicated by side reactions of the photoinitiator that can generate reactive species that contribute to cell toxicity, as shown in previous studies. Thus, other methods are required and led to the investigation of chemical crosslinking of collagen-based matrices with a combination of EDC and NHS. This method has been shown to increase mechanical stiffness of materials,[11,12,16,20,27,28] including organized tissues, without cytotoxic effects.

We investigated this method alone and in combination with photocrosslinking as a method for increasing collagen hydrogel stiffness. Experiments using EDC/NHS crosslinking alone on collagen gels were disappointing. Constructs exposed to this treatment were only loosely organized and collapsed under their own weight after being ejected from the mold. These gels could not be subjected to storage modulus evaluation. Despite a lack of structural integrity, these gels proved highly resistant to collagenase digestion, showing that crosslinking did, in fact, occur. Since the clinical application requires implantation of snug-fitting gels with a defined geometry, crosslinking with EDC/NHS alone is not an option. However, when photocrosslinking was practiced prior to EDC/NHS treatment a stiffer gel construct was obtained that did retain its shape when extruded from the mold. Thus, a dual crosslinking paradigm has potential to provide a practical implant for focal defect repair.

Rheometry testing demonstrated that the stiffness (storage modulus) of the gels increased in dual crosslinked gels (117.6 ± 6.9) more than 5-fold from photochemically crosslinked gels (21.4 ± 1.8 Pa, Fig. 3). Previous results, showing the lack of significant difference between the storage modulus of noncrosslinked, spontaneous collagen gels (25.8 ± 1.5 Pa) and photochemically crosslinked gels, were also confirmed as a *t*-test results in $p = 0.58$ between these two groups.

We attribute the resulting increase in G' of dual-crosslinked hydrogels to the addition of new chemical crosslinks throughout the collagen matrix with a resulting increase in crosslinking density. The 5-fold increase in G' by EDC/NHS on prior photocrosslinked hydrogels, in comparison to the null effect of EDC/NHS on spontaneously formed collagen gels discussed earlier, is an interesting observation. A possible explanation is that photocrosslinking treatment provides a stabilizing effect to the hydrogel, aligning collagen molecules to make the EDC/NHS crosslinking more efficient. EDC/NHS crosslinking increases stiffness of structured tissues, such as amnion,[11] tendon,[28] and sheep dermis.[29] Therefore, forming a more organized collagen matrix by photocrosslinking before exposure to EDC/NHS can positively affect the G' of the hydrogel.

Any crosslinking paradigm for ultimate clinical use cannot be toxic to the encapsulated cells. It is clear that chondrocytes are viable after encapsulation in the dual-crosslinked matrix (Fig. 5). Encapsulation of chondrocytes in type I collagen hydrogels, both spontaneous and photocrosslinked, was shown to be nontoxic in previous studies. The dual-crosslinked constructs were shown to be $96 \pm 2\%$ viable, well above the threshold of 90% viability for good ECM generation capacity used in previous studies.[10] Dead cells were few in number and confined to the outer extremes of the construct. Cells also appeared to adhere well to the dual-crosslinked matrix.

The gels that underwent dual crosslinking also retarded degradation by collagenase digestion (Fig. 1). Protection from proteases is important for matrix stability during early-stage chondrocyte viability and ECM generation, but over time the initial matrix must be remodeled by natural enzymatic activity and deposition of new hyaline cartilage ECM. If not, the crosslinked type I collagen matrix will impede production of neocartilage. We anticipate that there will be an optimum crosslinking level that provides sufficient stiffness to the gel but also allows for gradual enzymatic digestion and remodeling *in vivo*. We have shown by using different concentrations of EDC/NHS or treatment times that we can fine-tune the stiffness and also the degradation rate. Although collagenase degradation is affected by exposing the hydrogel to even the most dilute EDC/NHS groups, there is no significant effect on storage modulus until the hydrogel is exposed to higher concentrations. Compared to full crosslinking (1 h exposure time and 33 m*M* EDC/6 m*M* NHS) the stiffness of the gel increased at lower

concentrations and lower times of exposure (Figs. 3 and 4). Further studies are planned to investigate the cartilage generation capacity of encapsulated chondrocytes in gels in these groups.

A major concern in articular cartilage engineering is the ability of a construct to withstand biocompressive forces in the knee, which can be up to 3.40 ± 0.18 times patient bodyweight during a normal walking gait.[30] The increase in storage modulus after dual-crosslinking treatment was a very positive result. Creating a construct that has a higher storage modulus, (stiffer) would be beneficial as long as the increased stiffness does not impede neocartilage deposition within the matrix. Using a construct similar to those tested here may lead to shorter patient immobilization periods, shorter post-operative physical therapy periods, and an overall faster recovery when compared with recovery periods after solution-based cartilage reparative procedures like ACI. The proven viability of encapsulated chondrocytes and the protection against rapid enzymatic degradation that is provided by this dual-crosslinking paradigm may offer a route to a new, matrix-assisted articular cartilage replacement system.

CONCLUSIONS

From these data we can conclude that photochemically crosslinking collagen gels increases their resistance to collagenase digestion 2-fold. Adding a chemical crosslinking step to the photochemical crosslinking makes them even more resistant to digestion with collagenase without compromising the cell viability. The storage modulus of dual-crosslinked constructs was increased 5-fold over that of both photocrosslinked and spontaneous gels. Thus, the photochemical crosslinking, with or without chemical crosslinking, could resist degradation *in vivo* and be used as a scaffold for delivery of chondrocytes into cartilage defects. Changing the crosslinking strategy can improve the stiffness of the gel to provide additional stability to the gels when used *in vivo*.

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Covalently tethered TGF-β1 with encapsulated chondrocytes in a PEG hydrogel system enhances extracellular matrix production

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Abstract

Healing articular cartilage defects remains a significant clinical challenge because of its limited capacity for self-repair. While delivery of autologous chondrocytes to cartilage defects has received growing interest, combining cell-based therapies with growth factor delivery that can locally signal cells and promote their function is often advantageous. We have previously shown that PEG thiol-ene hydrogels permit covalent attachment of growth factors. However, it is not well known if embedded chondrocytes respond to tethered signals over a long period. Here, chondrocytes were encapsulated in PEG hydrogels functionalized with transforming growth factor-beta 1 (TGF-β1) with the goal of increasing proliferation and matrix production. Tethered TGF-β1 was found to be distributed homogenously throughout the gel, and its bioactivity was confirmed with a TGF-β1 responsive reporter cell line. Relative to solubly delivered TGF-β1, chondrocytes presented with immobilized TGF-β1 showed significantly increased DNA content and GAG and collagen production over 28 days, while maintaining markers of articular cartilage. These results indicate the potential of thiol-ene chemistry to covalently conjugate TGF-β1 to PEG to locally influence chondrocyte function over 4 weeks. Scaffolds with other or multiple tethered growth factors may prove broadly useful in the design of chondrocyte delivery vehicles for cartilage tissue engineering applications.

Keywords: cartilage tissue engineering, chondrocytes, protein conjugation, hydrogels, transforming growth factor-β1

1. Introduction

Healing articular cartilage defects remains a significant clinical challenge because of its limited capacity for self-repair and mechanical properties that are difficult to emulate.[1] Articular cartilage is an avascular tissue with a sparse population of cells surrounded by an extracellular matrix (ECM) that is regulated by numerous growth factors.[2] Therefore, tissue engineering strategies involving chondrocytes and growth factor delivery may help to improve the treatment of articular cartilage lesions.[3,4]

There is growing interest in the regenerative medicine community in methods to sequester and present bioactive therapeutic proteins to chondrocytes immobilized in three-dimensional matrices,.[5] Cytokines are attractive targets for tissue engineering since, at low concentrations, they can regulate cellular functions, such as proliferation and matrix production.[6] Many of these proteins are commonly introduced as soluble factors in culture media during *in vitro* experiments; however, *in vivo*, growth factors tend to be sequestered in the extracellular matrix, allowing local presentation to cells.[5]

A variety of natural and synthetic materials have been examined as potential cell carriers or as therapeutic agents for cartilage repair.[7,8,9] Hydrogel scaffolds appear to be one promising class of materials, due to their high water content which mimics native tissue microenvironments.[10] Furthermore, poly-(ethylene glycol) (PEG) hydrogels have been used to improve micofracture cartilage regeneration outcomes in human trials.[11]

Hydrogel systems permit sequestration of growth factors via covalent tethering, which can provide advantages compared to other forms of protein delivery. In particular, growth factors are typically cross-reactive with multiple cell types and can have short serum half-lives *in vivo*,

limitations that often necessitate localized presentation.[12] Since diffusion of lower molecular weight proteins in hydrogels can be quite rapid, some researchers have used microparticles for controlled release presentation of growth factors to encapsulated chondrocytes.[13] While this approach is quite useful, the process can increase the complexity of scaffold preparation and design. Variability can result from differences in protein loading, release kinetics, as well as the size distribution of loaded microparticles. Therefore, strategies to immobilize growth factors in a bioactive, physiologically relevant context are a complementary and important step towards directing cells to regenerate cartilage tissue.

As one robust method to create protein functionalized materials, we used thiol-ene chemistry to incorporate thiolated proteins in PEG hydrogels..Previously, PEG systems have been broadly explored for cell delivery applications.[14,15,16,17] Specifically, we formed PEG hydrogels through a photoinitiated step-growth polymerization, by reacting norbornene-terminated PEG macromolecules with a dithiol PEG crosslinker.[18] This photopolymerizable system allows for precise spatial and temporal control over polymer formation, as well as facile encapsulation of cells and biologics. The resulting crosslinked PEG hydrogel has been employed to encapsulate numerous primary cells with high survival rates following photoencapsulation.[10,19]

Previously, our group has successfully incorporated thiolated TGF-β1 in a chain-growth polymerized PEG diacrylate system and showed enhanced chondrogenesis of human mesenchymal stem cells (MSC).[20] Here, we encapsulated chondrocytes in step-growth polymerize PEG thiol-ene hydrogels, and we hypothesized that local presentation of TGF-β1 would influence chondrocyte secretory properties and improve the system's application for cartilage regeneration. Step-growth polymerization leads to more ideal network structures than chain-growth polymerization, and the thiol-ene chemistry has also been shown to be more

compatible for coupling proteins and maintaining their activity.[21] In contrast to other cell types,primary chondrocytes are a versatile cell source since they deposit a matrix more similar to articular cartilage. For example, MSC derived fibrocartilage is biomechanically inferior.[22] Additionally, a recent comparison study revealed that encapsulating chondrocytes in a PEG thiol-ene system yielded more hyaline-like cartilage than cells encapsulated in a PEG diacrylate system.[23]

In this work TGF-β1 was thiolated and incorporated into a PEG thiol-ene hydrogel. We selected TGF-β1 because it has been shown to increase chondrocyte proliferation and cartilage ECM production in both 3D[13] and 2D studies.[24] We confirmed the presence of tethered TGF-β1 in the gel by ELISA and investigated its bioactivity using a PE-25 cell reporter assay for SMAD2 signaling.[25] We also found that tethering growth factors to a scaffold results in increased cell proliferation and ECM production *in vitro*. These results suggest that a step-growth PEG hydrogel system is capable oftunable control of local bioactive signals. Chondrocytes encapsulated in this system are presented with a local and sustained delivery of TGF-β1, resulting in enhanced cartilage tissue regeneration.

2. Materials and Methods

PEG monomer synthesis

8-arm polyethylene glycol (PEG) amine norbornene M_n 10,000 was synthesized as previously described.[16] Briefly, 5-norbornene-2-carboxylic acid (predominantly endo isomer, Sigma Aldrich) was first converted to a dinorbornene anhydride using N,N'-dicyclohexylcarbodiimide (0.5 molar eq. to norbornene, Sigma Aldrich) in dichloromethane. The 8-arm PEG monomer (JenKem Technology USA) was then reacted overnight with the norbornene anhydride (5 molar eq. to PEG hydroxyls) in dichloromethane. Pyridine (5 molar eq. to PEG hydroxyls) and 4-

dimethylamino pyridine (0.05 molar eq. to PEG hydroxyls) were also included. The reaction was conducted at room temperature under argon. End group functionalization was verified by ^{1}H NMR to be >90%. ^{1}H NMR (500 MHz,$CDCl_3$) δ 6.30-5.80 (m,16H), 4.0-3.0 (m,1010H), 2.5-1.2 (m,100H). The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as described.[19] The 3.5 kDa PEG dithiol linker was purchased from JenKem Technology.

Cell harvest and expansion

Primary chondrocytes were isolated from articular cartilage of the femoral-patellar groove of 6 month old Yorkshire swine as detailed previously.[26] Cells were grown in a culture flask in media as previously described.[27] Briefly, cells were grown in DMEM growth medium (phenol red, high glucose DMEM supplemented with ITS+Premix 1% v/v (BD Biosciences), 50 μg/mL L-ascorbic acid 2-phosphate, 40 μg/mL L-proline, 0.1 μM dexamethasone, 110 μg/mL pyruvate, and 1% penicillin-streptomycin-fungizone with the addition of 10 ng/mL IGF-1 (Peprotech) to maintain cells in de-differentiated state. ITS promotes formation of hyaline cartilage over serum.[28] Cultures were maintained at 5% CO_2 and 37 $^{\circ}$C.

Mink lung epithelial PE-25 cells containing a stably transfected luciferase reporter gene for TGF-β1 were cultured in low glucose DMEM supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin-fungizone. Cells that were passaged three times were used in encapsulation experiments.

PEG hydrogel polymerization and growth factor incorporation

2-Iminothiolane (Pierce) was used to thiolate human TGF-β1 (Peprotech). Briefly, 2-Iminothiolane was reacted at a 4:1 molar ratio to TGFβ for 1 hour at RT. Thiolated TGFβ was pre-reacted at various concentrations with PEG norbornene monomer solution prior to cross-

linking via photoinitiated polymerization with UV light (I_o~3.5 mW/cm^2 at λ=365 nm) and 0.05 wt% LAP for 30 s. The monomer solution was then crosslinked with a 3.5 kDa PEG dithiol at a stoichiometric ratio of [40 mM dithiol]: [80 mM Norbornene] in a 10 wt% PEG solution using longwave ultraviolet light (I_o~3.5 mW/cm^2 at λ=365 nm) for 30 s. (Scheme 1)

Quantifying growth factor incorporation

10 wt% hydrogels were synthesized with tethered TGF-β1 at 0, 10, 50, or 90 nM and prepared for cryosectioning as previously described.[29] Briefly, hydrogels were flash frozen in liquid nitrogen and placed in HistoPrep (Fisher Scientific) in cryomolds. 20 μm cross-sections along the plane of the construct were collected on SuperFrost® Plus Gold slides (Fisher Scientific).

40 μL disc-shaped gels (O.D. ~5 mm, thickness ~ 2 mm) without encapsulated cells and with varying concentrations of tethered growth factor were also prepared and sectioned. 20 μm sections were collected from the top, middle, and bottom of gel. To quantify the TGF-β concentration in each section, a modified ELISA was used as previously described.[14] Briefly, sections were blocked for 1 hour at RT in 5% bovine serum albumin (BSA). Sections were washed 3x in ELISA buffer (0.01% BSA, & 0.05% Tween-20 in PBS) prior to incubation with a mouse anti-human TGF-β1 antibody (Peprotech) at 1:100 dilution overnight at 4 ^{0}C. Sections were washed again, then incubated with goat anti mouse–HRP (eBioscience) for 1 hour at RT and washed again. Sections were incubated with 100 μL of peroxidase and 3,3',5,5' tetramethylbenzidine substrate until color developed then the reaction was stopped using 100 μL 2 N sulfuric acid. The absorbance was measured at 450 nm using a Bio-Tek H1 spectrophotometer.

To calculate the theoretical loading of growth factor in each section, the volume was determined assuming the section was a thin disc with a 5 mm diameter and 20 μm height. Using $V = \pi r^2 h$

and the molecular weight of TGF-β1 (M_n=25,000 g/mol), the amount of growth factor per section was calculated in nanograms. For instance, a 50 nM 40 μL gel section is expected to have 0.5 ng of TGF-β1 per 20 μm section assuming ideal conditions.

Finally, a standard curve was made simultaneously by prepping 96 well high binding clear plates with known amounts of TGF-β1. The 0 nM value at 450 nm absorbance was subtracted out from all values in the curve.

TGF-β1 bioactivity and cellular signaling

PE-25 cells were encapsulated in 10 wt% gels functionalized with a 1 mM Cys-Arg-Gly-Asp-Ser (CRGDS) peptide to promote survival. Thiolated TGF-β1 was incorporated into the gel at 0, 12.5, 25, 50, or 100 nM. Additionally, cells encapsulated in PEG gels without tethered growth factor were exposed to soluble TGF-β1 at concentrations of 0, 0.2, 0.3, 1, or 2 nM. Cells were photo-encapsulated at a density of 40 million cells/mL, and cell-laden hydrogels were formed in syringe tips at a volume of 40 μL. Following encapsulation, hydrogels were placed into DMEM growth medium in 48 well plates and incubated overnight at 37 $^{\circ}$C, 5% CO_2. Afterwards, hydrogels were incubated in Glo-Lysis buffer (Promega) for 10 min at 37 $^{\circ}$C; the samples were centrifuged for 10 min (13,400 rpm.4 $^{\circ}$C), and the lysate was transferred to white 96 well plates (50 μL per well). 50 μL luciferase substrate (Promega) was added to the lysate for 5 min and luminescence was quantified between 300-700 nm.

Chondrocyte encapsulation in PEG thiol-ene hydrogels

Chondrocytes were encapsulated at 40 million cells/mL in 10 wt% monomer solution and thiolated TGF-β1 at concentrations of 0 or 50 nM. 40 μL cell-laden gels were immediately placed in 1 mL DMEM growth medium (without phenol red) in 48 well non-treated tissue culture plates. As a positive control, a subset group of gels without tethered growth factor was

exposed to 0.3 nM (7.5 ng/mL) soluble TGF-β1. Media was changed every 3 days. Samples were collected at days 1, 14, and 28 for analysis of ECM production and chondrocyte proliferation. At day 1 and 28 cell viability was assessed using a LIVE/DEAD® membrane integrity assay and confocal microscopy.

Biochemical analysis of cell-hydrogel constructs

Cell-laden hydrogels were collected at specified time points, snap frozen in LN_2, and stored at -70 °C until analysis. Hydrogels were digested in enzyme buffer (125 μg/mL papain [Worthington Biochemical], and 10 mM cysteine) and homogenized using 5 mm steel beads in a TissueLyser (Qiagen). Homogenized samples were digested overnight at 60 °C.

DNA content was measured using a Picogreen assay (Invitrogen). Cell number was determined by assuming each cell produced 7.7 pg DNA per chondrocyte.[30] Sulfated glycosaminoglycan (GAG) content was assessed using a dimethyl methylene blue assay as previously described with results presented in equivalents of chondroitin sulfate.[31] Collagen content in the gels was measured using a hydroxyproline assay, where hydroxyproline is assumed to make up 10% of collagen.[32] DNA content was normalized per gel while GAG and collagen content were normalized per cell.

Histological and immunohistochemical analysis

On day 28, constructs (n=2) were fixed in 10% formalin for 30 min at RT, then snap frozen and cryosectioned. Sections were stained for safranin-O or masson's trichrome on a Leica autostainer XL and imaged in bright field (40X objective) on a Nikon inverted microscope.

For immunostaining, sections were blocked with 10% goat serum, then analyzed by anti-collagen type II (1:50, US Biologicals) and anti-collagen type I (1:50). Sections were treated with appropriate enzymes for 1 hour at 37 °C: hyaluronidase (2080 U) for collagen II, and pepsin A

(4000 U) with Retrievagen A (BD Biosciences) treatment for collagen I to help expose the antigen. Sections were probed with AlexaFluor 568-conjugated secondary antibodies and counterstained with DAPI for cell nuclei. All samples were processed at the same time to minimize sample-to-sample variation. Images were collected on a Zeiss LSM710 scanning confocal microscope with a 20X objective using the same settings and post-processing for all images. The background gain was set to negative controls on blank sections that received the same treatment. Positive controls were performed on porcine hyaline cartilage for collagen type II and porcine meniscus for collagen type I (Supplemental Figure 1).

Statistical Analyses

Data are shown as mean ± standard deviation. Two way analysis of variance (ANOVA) with Bonferroni posttest for pairwise comparisons was used to evaluate the statistical significance of data. One way ANOVA was used to assess differences within specific conditions. $p < 0.05$ was considered to be statistically significant.

3. Results

Distribution of thiolated TGF-β1 in PEG hydrogels

We confirmed that TGF-β1 was homogenously distributed within the gel after the thiol-ene tethering process, using a modified section ELISA.[14] The results presented in Figure 1 show TGF-β1 incorporation throughout the gel, and its relatively homogeneous distribution among gel regions. We further showed that experimentally measured values were similar to theoretically calculated levels (0.1 ng for 10 nM, 0.5 ng for 50 nM, and 0.9 ng for 90 nM).

Bioactivity and concentration of tethered TGF-β1 in 3D culture

We investigated the bioactivity of tethered TGF-β1 in 3D culture using a reporter cell line. Briefly, it was shown that tethered proteins typically maintain high levels of bioactivity when conjugated using thiol-ene reactions.[20] We further determined concentrations of soluble and tethered TGF-β1 that yielded a maximal response in PE-25 cells at a seeding density of 40 million cells/mL. In Figure 2 a, there was a significant difference in luciferase output of 50 nM gels compared to other conditions. In Figure 2 b, 0.3 nM via soluble delivery elicited a maximal cellular response. Interestingly, when we dosed 50 nM of soluble TGF β-1 to encapsulated PE-25s at 40 million cells/mL, the average luciferase response was ~ 6,510 arbitrary units (n=4), which is a 3-fold lower response than for the same concentration of tethered TGF-β1. Based on these results, we elected to dose soluble TGF-β1 at the magnitude of 0.3 nM. Overall, these results suggest that tethered TGF-β1 is bioactive, and at 40 million cells/mL, the conditions that elicited the highest response to TGF-β1 were 0.3 nM (soluble) and 50 nM (tethered).

Proliferation of chondrocytes exposed to TGF-β1

Cell viability for all encapsulation and culture conditions was between 80%-90% assessed by live/dead membrane integrity assay at both days 1 and 28. Figure 3 a shows the rounded shape of encapsulated cells; there was significant increase in number of cells in the 50 nM TGF-β1 tethered gels. To further quantify this proliferation, we harvested samples at day 1, 14, and 28 and assayed for DNA content (Figure 3 b). There was a statistically significant increase in DNA content, at day 28, for cells encapsulated in 50 nM TGF-β1 containing gels. Further, there was significantly more DNA in the day 28 50 nM condition than either the 0.3 nM or 0 nM gel condition ($p<0.001$). Combined with the viability results, these data suggest an increase in chondrocyte proliferation in response to tethered growth factor presentation.

Matrix deposition as a function of TGF-β1 presentation and culture time

We assessed glycosaminoglycan (GAG) and total collagen content of gels at day 1, 14, and 28. Encapsulated chondrocytes were either exposed to 0 nM, 0.3 nM solubly or 50 nM tethered TGF-β1. Measured quantities were normalized to cell content in the respective hydrogel formulations.

In Figure 4 a, GAG production per cell on day 28 for the tethered construct was significantly higher than non-treated groups ($p<0.001$). There was also a significant difference at day 28 between constructs that presented tethered TGF-β1 compared to solubly delivered TGF-β1 ($p<0.05$), suggesting that the tethered growth factor enhanced ECM production over soluble growth factor delivered in the media.

In Figure 4 b, total collagen production per cell was highest at day 28 from the construct with tethered TGF-β1. Further, there was a significant difference between the tethered and soluble TGF-β1 conditions ($p<0.01$) at day 28, and the tethered group was significantly increased from the 0 nM group ($p<0.001$), indicating that collagen content is highest in the tethered protein constructs.

Matrix organization

We examined the distribution and deposition of extracellular matrix molecules by histological and immunofluorescence techniques. Masson's trichrome staining (Figure 5 a,c,e) revealed collagen deposition increased in the pericellular space of encapsulated chondrocytes with both tethered and soluble TGF-β1 gels on day 28 compared to 0 nM gels. Overall, it appears that most of the pericellular collagen deposition occurs in the 50 nM gels at day 28. In a similar fashion, safranin-O (Figure 5 b,d,f) staining revealed that GAG deposition localized in the pericellular

region with increased deposition per cell in the presence of TGF-β1. These results support the data that tethered TGF-β increases ECM secretion.

Immunofluorescence staining revealed that by day 28, there was a scarce amount of collagen I throughout all samples (Figure 6 a,c,e) and that collagen II was prevalent in the growth factor treated samples (Figure 6 d,f) compared to the 0 nM sample (Figure 6 b). A high collagen II and low collagen I signal is indicative of articular cartilage, and the constructs maintained that phenotype over 28 days of culture.[33]

4. Discussion

Engineering a clinically viable scaffold for chondrocyte delivery and promotion of cartilage regeneration is challenging, partly because of the time required for chondrocytes to generate a robust matrix. By encapsulating chondrocytes in a PEG thiol-ene system with localized presentation of a growth factor, we have shown quantitatively and qualitatively, *in vitro,* that cells survive, proliferate, and generate cartilage specific ECM molecules at a higher rate than without the growth factor. Tethering growth factors into a synthetic material scaffold integrates the promoting effects of a protein cross-linked gel without gel to gel variability. A cell delivery system with such properties can provide certain advantages for clinical applications in techniques such as matrix assisted autologous chondrocyte transplantation (MACT).

There are many advantages to tethering growth factors into a gel system for tissue engineering purposes. Localized presentation precludes growth factors from activating unnecessary cell targets in an *in vivo* setting. Additionally, it requires a lower amount of growth factor. In this 28 day study, TGF-β1 is dosed in 1 mL media every 3 days at 0.3 nM that results in ~ 70 ng of protein delivered to the cell-laden gel. For the same time period and experimental conditions, a 50 nM tethered gel corresponds to ~50 ng of TGF-β1/gel, yet led to higher matrix production and

DNA content at day 28. When using an expensive and/or potent growth factor to promote tissue regeneration, a tethered system can potentially provide a more efficient and effective delivery system for long time periods appropriate for clinical settings.

In these studies, we chose to look specifically at chondrocytes encapsulated at 40 million cells/mL, since this cell density has been previously shown to be an optimal choice for *in vivo* studies with hydrogel delivery systems.[34,35,36] We used a cellular assay, based on PE-25 cells as a reporter system with a luciferase output, to determine that an effective concentration of growth factor to deliver to cells was 50 nM (Figure 2 a) for tethered TGF-β1 and 0.3 nM for soluble TGF-β1 (Figure 2 b). We chose the initial concentrations of TGF-β1 for the PE-25 experiments based on previous work for promoting chondrogenesis of hMSCs.[20] We hypothesized that encapsulated cells may not respond as well to higher concentrations of soluble TGF-β1 than tethered TGF-β1, because PE-25s may internalize the factor, and seeding at high density may reduce the cellular response. Related studies with Mv1Lu cells showed that they internalized TGF-β1, so it is reasonable to consider this explanation for the PE-25 experiments.[37]

We speculate that for gels presenting 100 nM of tethered TGF-β1, the PE-25s encapsulated at 40 million cells/mL showed less activity compared to 50 nM gels (Figure 2 a) because growth factors can have pleiotropic effects that may lead to a negative feedback loop. Additionally, since TGF-β binds to a dimer receptor, which requires two receptor subtypes to join to initiate the signaling cascade, it is possible that the orientation of growth factors around the cell prevents complete binding since both subtype receptors may be occupied by separate ligands when only one is required for signaling activation.[38]

We chose to use human TGF-β1 with porcine chondrocytes because the PE-25 system has already been established with human TGF-β1,[25] and porcine chondrocytes will be used in future pre-clinical animal studies. We believe that this is unlikely to affect the outcomes of our studies, since mature TGF-β1 is known to be highly conserved (>99% amino acid sequence identity) throughout mammalian species.[39]

The data presented in this study suggest that the PEG thiol-ene platform with tethered TGF-β represents a bioactive scaffold with potential tissue engineering applications for chondrocyte delivery. Chondrocytes maintained a spherical morphology, similar to native chondrocytes, in the gel over a 28 day period, as shown in Figure 3a, which suggests the cells are less likely to de-differentiate and generate hyaline-like cartilage.[40] Chondrocytes also increased in cell number when cultured in PEG thiol-ene gels as shown in Figure 3 b, and especially when TGF-β1 is presented, which is known to induce proliferation.[24] Porcine chondrocyte doubling time in 2D culture is around 6.4 ± 0.3 days in serum-containing media.[41] We speculate that part of the reason the cells did not double at a similar rate when encapsulated in the PEG gels is that the selected gel formulations are non-degradable. Thus, the polymer network limited the amount of space available for chondrocytes to grow, and the media did not contain serum. This result was confirmed by a study with rat chondrocytes grown in a non-degradable 3D scaffold which had a longer doubling time (10.04 ± 0.9 days) than cells grown in 2D (2.94 ± 0.3 days).[42]

Extracellular matrix production data revealed that over 28 days, the tethered-protein gel stimulated chondrocytes to produce more GAGs and collagen, as quantified in Figure 4. The cells maintained a high rate of ECM production even though matrix proteins accumulate around the cell after 28 days. This phenomenon implies that TGF-β1 may maintain activity and interact with the chondrocytes, despite the increased pericellular matrix. Furthermore, when compared to

a tethered TGFβ study investigating MSC chondrogenesis,[20] chondrocytes maintained a similar level of GAG production and also express collagen type II on a similar time scale.

A study with juvenile and adult chondrocytes encapsulated in degradable gels had higher GAG and collagen outputs per cell over a 28 day period compared to the ones in this study.[43] We expected that a degradable gel allows for greater ECM deposition as posited by various groups.[44,45]Additionally, histology and immunofluorescence staining confirmed that matrix was primarily deposited pericellularly in all conditions, but at a higher level in gels with tethered TGF-β1. While the secreted matrix was primarily confined to the pericellular region, there were some areas where the ECM molecules, especially GAGs, were more dispersed between cells (Figure 5). These data suggest the need for tethering TGF-β1 to a degradable PEG thiol-ene system to enhance ECM production and elaboration, with the potential to better capture biochemical and biomechanical properties of native hyaline tissue.

Conclusion

We confirmed that thiol-ene reactions allow conjugation of TGF-β1 into PEG gels, while maintaining bioactivity and signaling to encapsulated cells. We showed that tethered TGF-β1 increased the proliferation rate and ECM production of chondrocytes over a 28 day period, at levels exceeding that of cells in gels where TGF-β1 was dosed in the culture medium or those that were untreated. The tethered TGF-β hydrogels utilized a lower total protein dosage while still promoting high levels of proliferation and matrix production of chondrocytes. Furthermore, chondrocytes maintained a spherical morphology in the thiol-ene PEG gels with high viability and a phenotype that resembles articular cartilage (i.e. high collagen II and low collagen I levels). Collectively, these results demonstrate the feasibility of delivering bioactive protein

signals in a 3D culture platform to enhance matrix production of chondrocytes. This platform may have further applications as a scaffold for *in vivo* cartilage regeneration.

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Disclosure

No benefit of any kind will be received either directly or indirectly by the authors. The content of the manuscript does not necessarily reflect the position or policy of the Government, and no official endorsement should be inferred.

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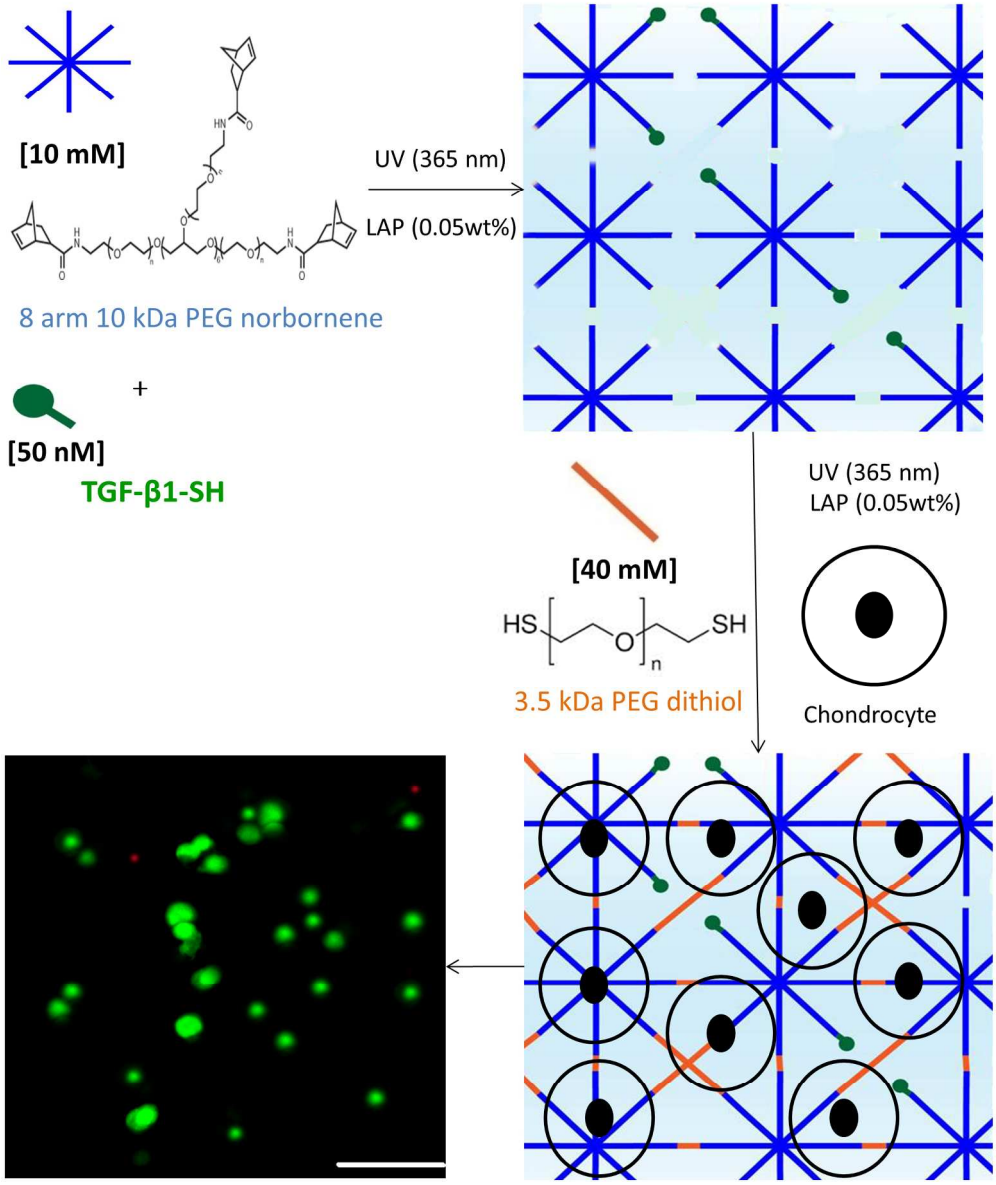

Scheme 1. Pre-polymerization scheme with thiolated TGF-β1. Initially thiolated TGF-β1 is phototethered into the 8 arm 10 kDa PEG norbornene network, then the 3.5 kDa dithiol crosslinker is added in with chondrocytes to complete the encapsulation process. Growth factor is not drawn to scale. In featured experiments, there is a lower amount of growth factor attached to the monomer end. Chondrocytes seeded at 40 million cells/mL retain a rounded morphology similar to cells in native tissue. Scale bar represents 50 μm.
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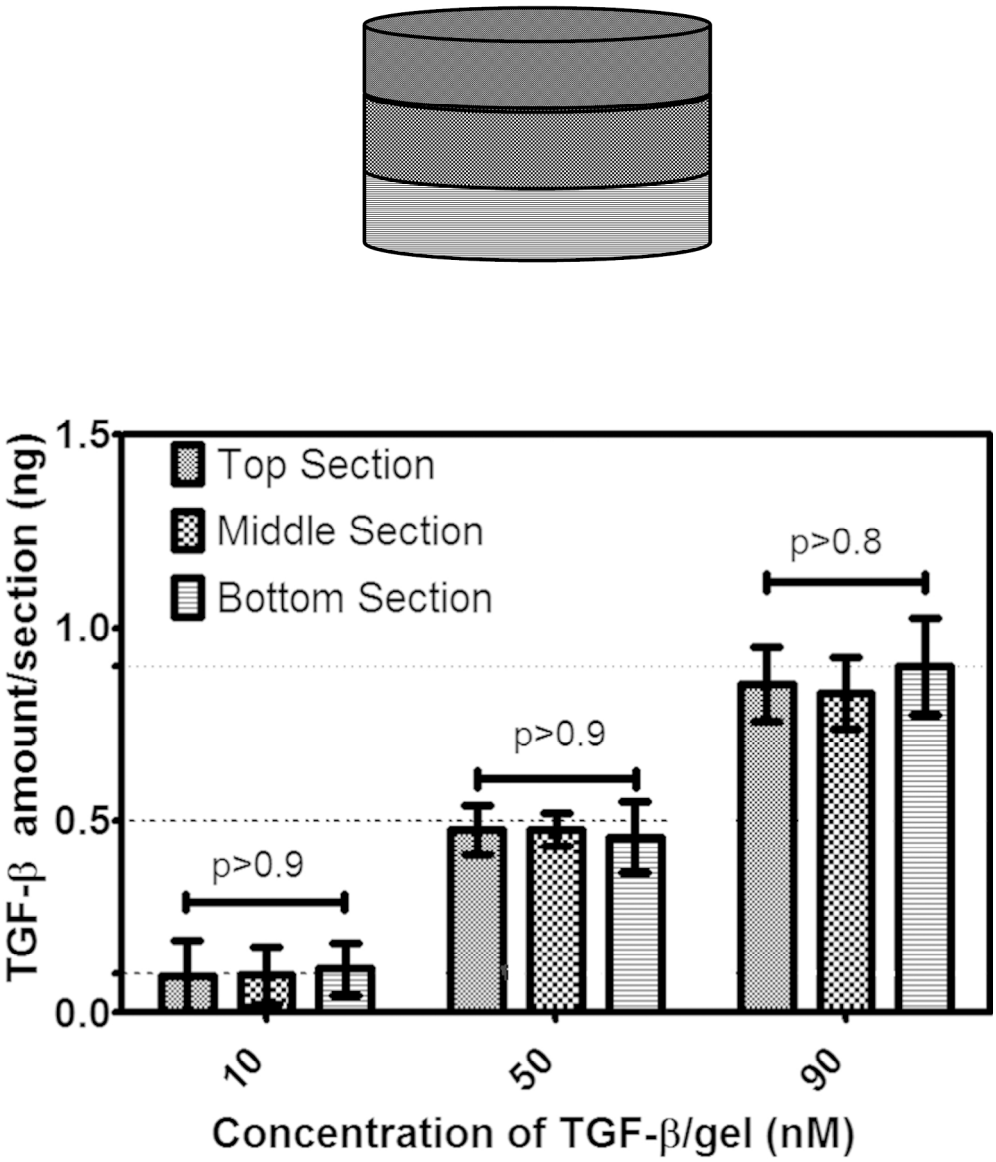
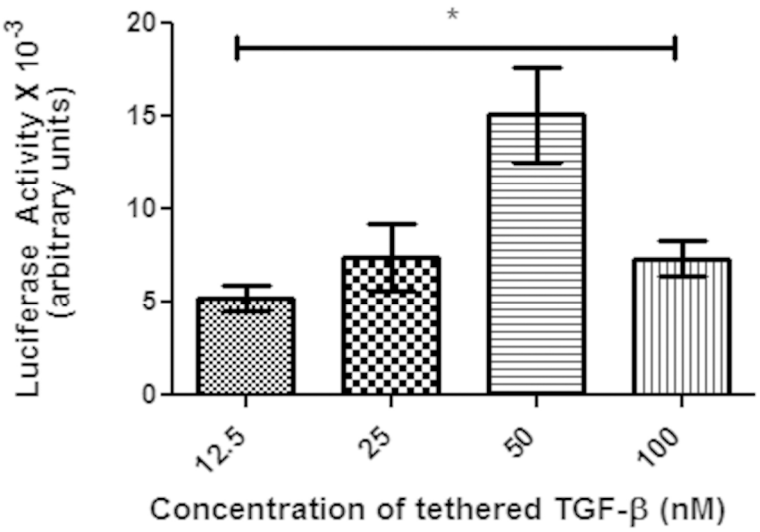


Figure 1. TGF-β1 is homogenously distributed throughout the PEG hydrogel. Section ELISA of tethered gels without cells show detection of TGF-β at similar levels to theoretical values with graphic on top depicting slice areas. Each section ~ 20μm thickness. Theoretical values indicated by dashed lines (0.1 ng for 10 nM, 0.5 ng for 50 nM, and 0.9 ng for 90 nM gels). 0 nM value is subtracted out of all conditions. Results are presented as mean activity ± s.d. (n=2). Solid lines indicate p values with one way ANOVA analysis to confirm sections of each gel are not statistically different from each other.
183x214mm (300 x 300 DPI)

a.



b.

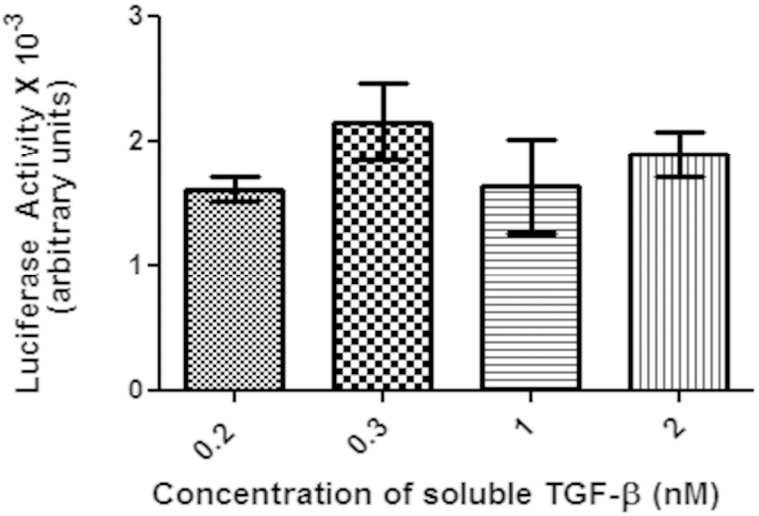


Figure 2. Determining TGF-β1 concentration that yields maximal response. (a) PE-25s were encapsulated at 40 million cells/mL with varying concentrations of tethered TGF-β and 50 nM yielded a maximal response. * indicates statistically significant difference between 50 nM and the other concentrations with $p<0.001$. Results are presented as mean activity ± s.d. (n=4). (b) PE-25 cells encapsulated at 40 million cells/mL were transiently exposed to varying concentrations of TGF-β in the media. The 0.3 nM output is higher on average than the other concentrations. Results are presented as mean activity ± s.d. (n=4).
161x230mm (300 x 300 DPI)

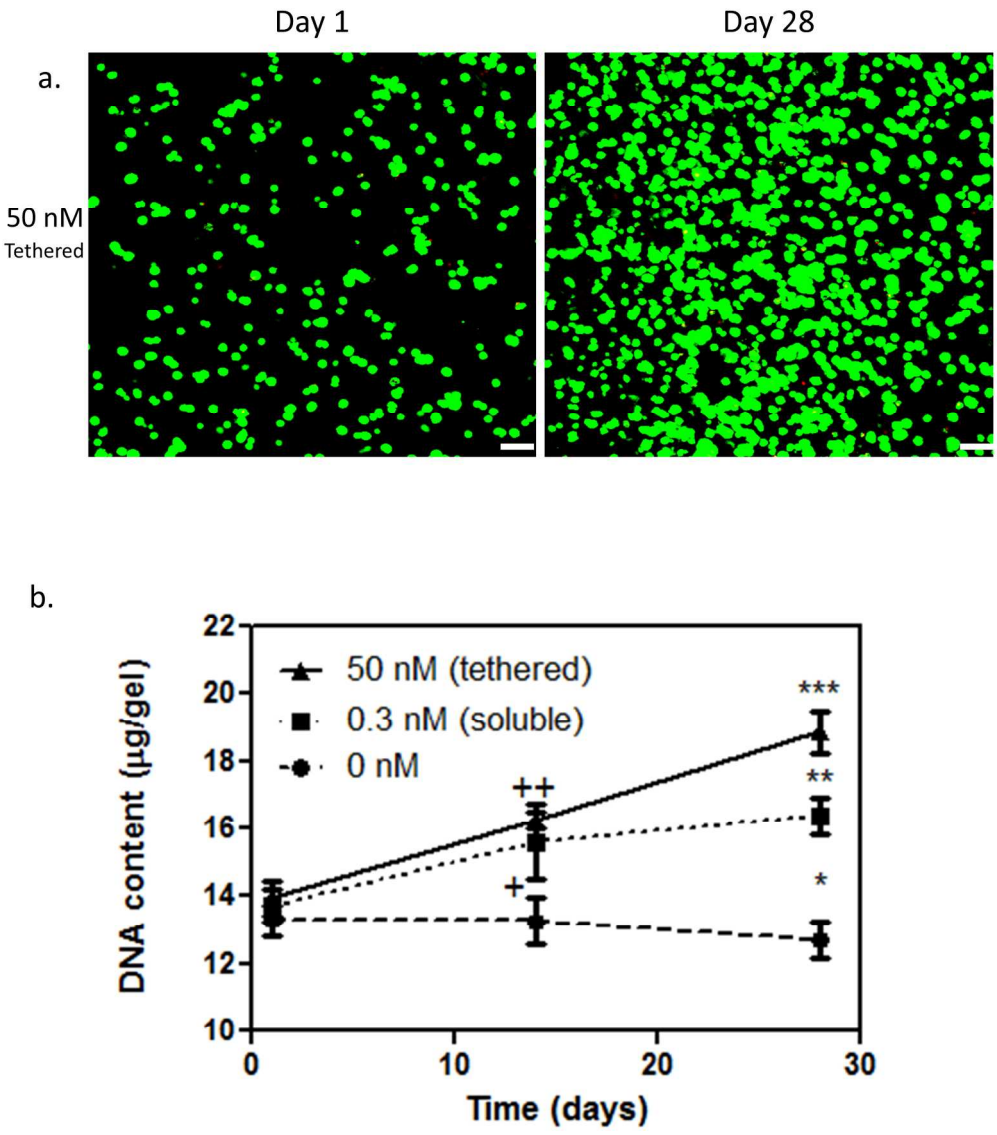
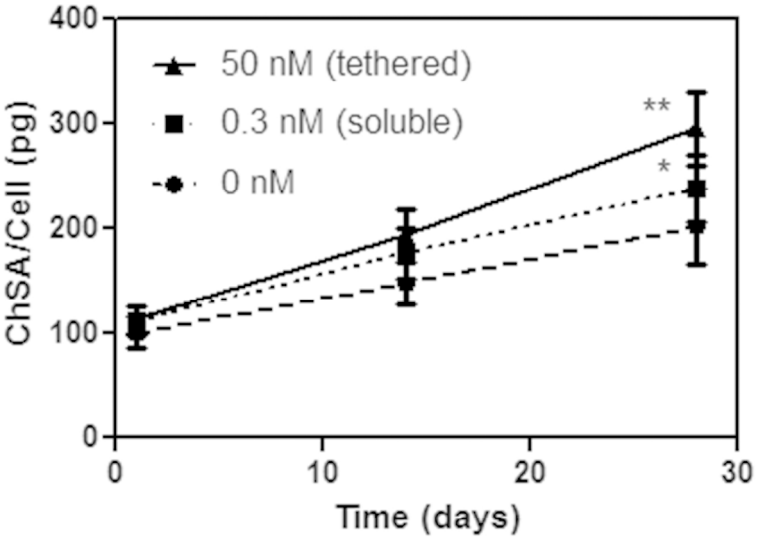


Figure 3. Increased proliferation of chondrocytes exposed to TGF-β1.(a) Live/Dead staining of 50 nM gels seeded at 40 million cells/mL on day 1, and day 28 shows chondrocytes retain a spherical morphology, have high viability, and increase in number. Scale bars represent 50 µm. (b) DNA content of chondrocytes encapsulated at 40 million cells/mL that were exposed to 0 nM, 0.3 nM which was delivered through the media, or 50 nM which was tethered into the gel. Over a 28 day period, the cells in the 50 nM condition show a steady rate of increase of DNA content. + indicates significant difference between the 0.3 nM and 0 nM case ($p<0.001$), ++ indicates significant difference between 50 nM and 0 nM case ($p<0.001$), * indicates significant difference between 0.3 nM and 0 nM ($p<0.001$), ** indicates significant difference between 50 nM and 0.3 nM case at day 28 ($p<0.001$), and *** indicates significant difference between 50 nM and 0 nM for day 28 ($p<0.001$). Results are presented as mean± s.d. (n=3).
190x214mm (300 x 300 DPI)

a.



b.

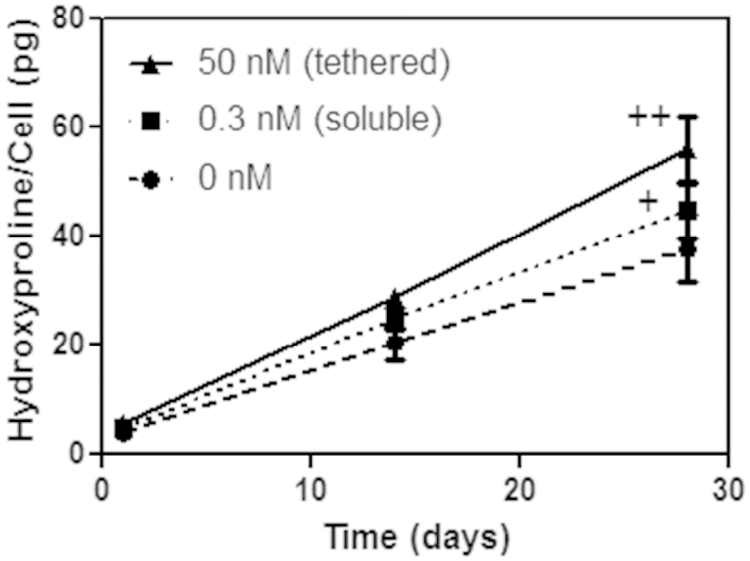


Figure 4. Enhanced matrix production of encapsulated chondrocytes exposed to TGF-β. (a) GAG production was normalized per cell. * indicates significant difference between 50 nM and 0.3 nM condition at day 28 ($p<0.05$), ** indicates significant difference between 50 nM and 0 nM at day 28 ($p<0.001$). Data presented as mean ± s.d. (n=3). (b) Collagen production was normalized per cell. + indicates significant difference between 50 nM and 0.3 nM at day 28 ($p<0.01$) and ++ indicates significant difference between 50 nM and 0 nM at day 28 ($p<0.001$). Data presented as mean ± s.d. (n=3).
164x217mm (300 x 300 DPI)

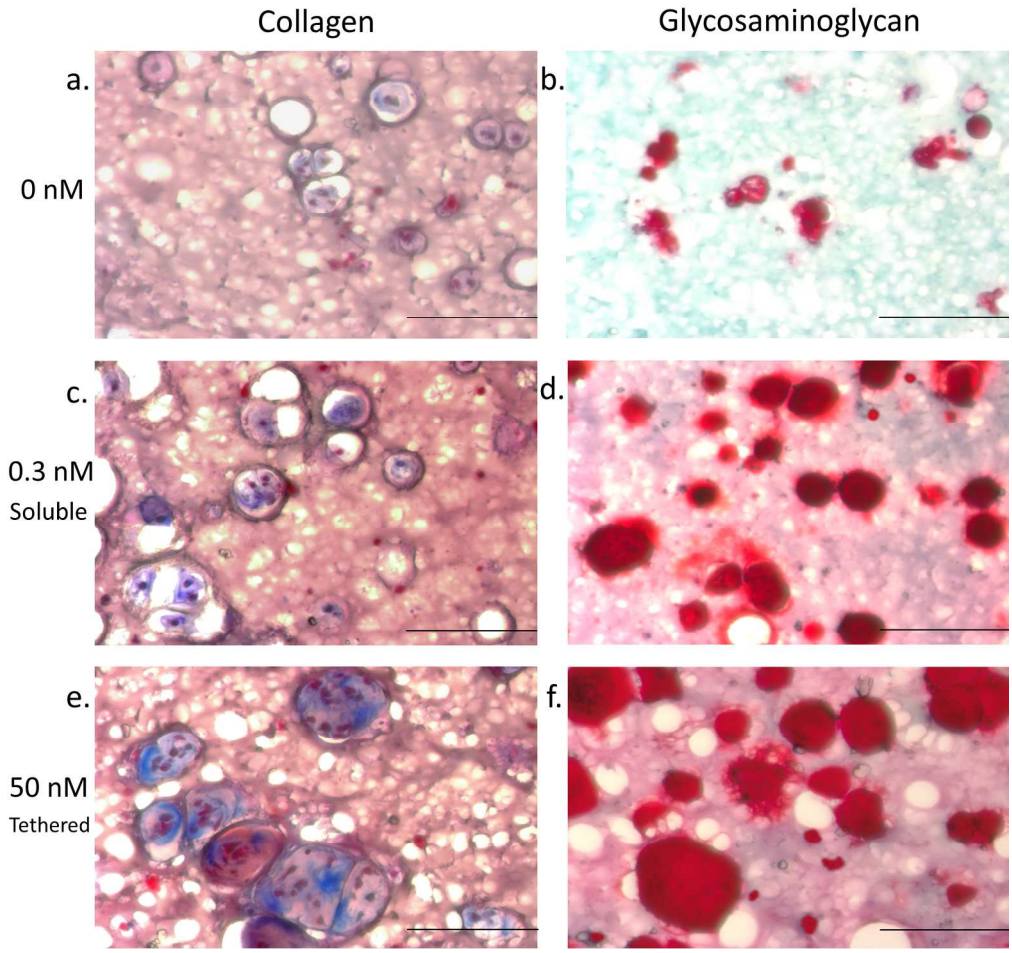


Figure 5. Matrix protein distribution in gels. At day 28, gels seeded with chondrocytes at 40 million cells/mL were sectioned and stained for matrix distribution. (a) 0 nM gel stained for collagen, (b) 0 nM gel stained for GAG, (c) 0.3 nM (soluble) gel stained for collagen, (d) 0.3 nM (soluble) gel stained for GAG, (e) 50 nM (tethered) gel stained for collagen, (f) 50 nM (tethered) gel stained for GAG. Blue indicates collagen and red indicates GAG. Scale bars represent 100 μm.
187x177mm (300 x 300 DPI)

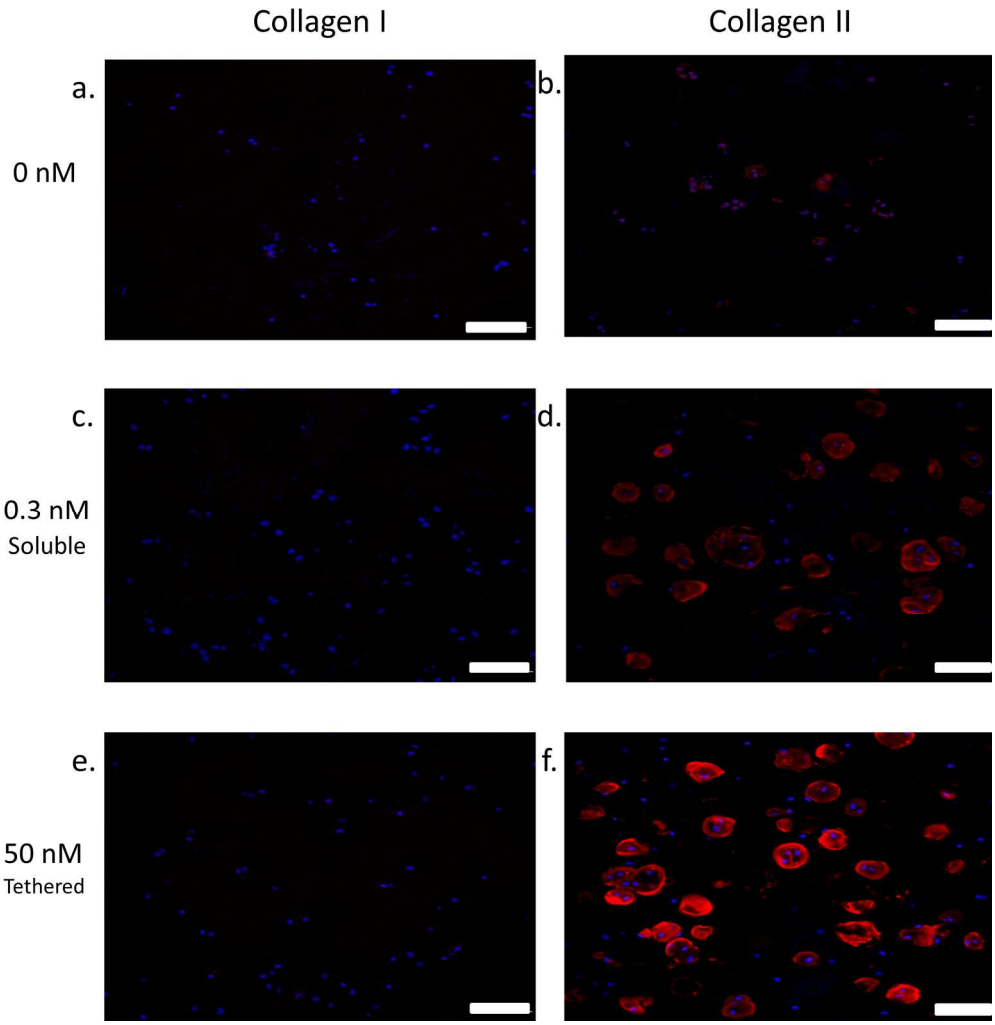
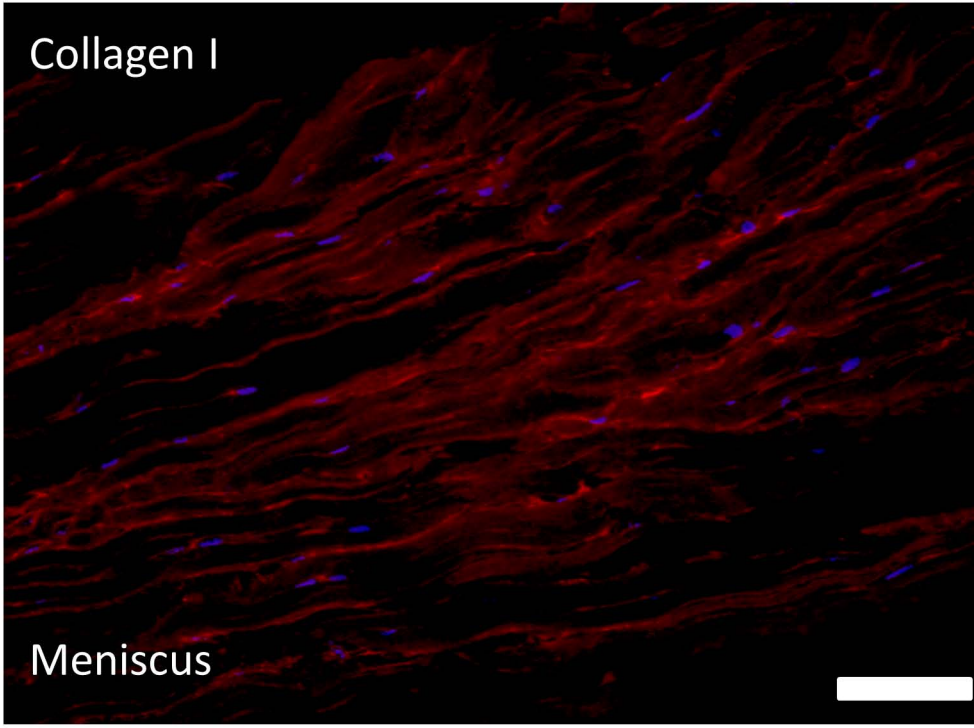


Figure 6. Collagen I vs. collagen II distribution in constructs. Gels seeded with chondrocytes at 40 million cells/mL were cryosectioned at day 28. Immunohistochemistry analysis reveals collagen type distribution in scaffolds. (a) 0 nM with collagen I, (b) 0 nM with collagen II, (c) 0.3 nM (soluble) with collagen I, (d) 0.3 nM (soluble) with collagen II, (e) 50 nM (tethered) with collagen I, (f) 50 nM (tethered) with collagen II. Sections were stained red for both anti-collagen I and anti-collagen II antibodies and were counterstained with DAPI (blue) for cell nuclei. Scale bars represent 50 μm.
187x190mm (300 x 300 DPI)

a.



b.